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13. ABSTRACT (Maximum 200 Words)
The goal of this Academic Award is to appraise critically the state of dietary prevention of breast cancer and to forge new avenues of investigation in the field of nutrition. This goal consists of two specific aims: (1) to determine the importance of timing of specific dietary exposures on breast cancer risk; and (2) to develop a novel nutrition and cancer program to the PI's institution. Following achievements have been made towards the aims: **(1)** the PI's laboratory is currently focusing on investigating the impact of an exposure, either during *in utero* period through a pregnant dam, during prepubertal period or during pregnancy, to different types of dietary fats, phytoestrogens and alcohol on breast cancer risk. Since the beginning of the second year of the award period in 7/1/00, these studies have generated 6 published, 2 in press, and 2 submitted papers, and 4 manuscripts in preparation. The studies show that *in utero* exposure to n-6 or n-3 polyunsaturated fatty acids (PUFAs), genistein or soy may have opposite effects on mammary tumorigenesis than prepubertal exposures. Human studies have also been completed or in progress to determine whether the findings obtained in animal models are relevant to breast cancer prevention in women. **(2)** A new course (PI is the course director) entitled "Life style and cancer prevention" was taught for a first time as part of the Tumor Biology graduate program at Georgetown University. In addition, the PI has obtained a planning grant from NCI to develop a program consisting of several RO1 type awards to study the interactions among nutrition, genes and cancer.

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INTRODUCTION:

The purpose of Academic Award is to allow me to appraise critically the state of **dietary prevention of breast cancer** and to forge new avenues of investigation in the field of nutrition. These new avenues are achieved through studies that examine the role of diet during periods of rapid mammary cell proliferation, such as fetal life, puberty and pregnancy, in influencing breast development and breast cancer risk. In addition, studies will be done in human populations to investigate whether the data obtained using animal models apply to women. Dietary factors that are the focus of these studies are **polyunsaturated fatty acids, phytoestrogens, and alcohol**. Special emphasis is given to identify their mechanism of action. In particular, the role of the two estrogen receptor isotypes (ERα and ERβ) and eicosanoid pathways (cyclooxygenase, lipooxygenase and P450) in mediating the effects of PUFA, phytoestrogens and alcohol, are assessed. In women, intermediate biomarkers of breast cancer risk are studied in the nipple aspirate fluid (NAF). During the funding period, a course addressing critical nutritional issues in breast cancer, has been developed at the Georgetown University, Department of Oncology as a new initiative in the existing Tumor Biology program.

BODY:

Task-1. Effect of dietary manipulations occurring during sensitive periods, on breast cancer risk using animal models (months 1-24).

These dietary manipulations will occur during pregnancy and they include:
1.1. n-6 and n-3 PUFA
1.2. phytoestrogens
1.3. alcohol

Research accomplished associated with Task-1

The following studies have been completed, and the results which have either been published, in press, submitted or in preparation, are listed below. These papers report specific findings or summarize our data, putting it into the context of current state of dietary prevention of breast cancer. The latter is appropriate for the overall purpose of the academic award.

1.   HILAKIVI-CLARKE L, CHO E, DEASSIS S, OLIVO S, EALLEY E, BOUKER KB, WELCH J, KHAN G, CLARKE R, CABANES A. Maternal and prepubertal diet, mammary development, and breast cancer risk. *J Nutr* 131:154S-157S, 2001. (In appendix A)
2)   CLARKE R, HILAKIVI-CLARKE LA, TROCK B. Dietary and environmental sources of estrogenicity and breast cancer risk. *Biologist* 48:216, 2001. (In appendix A)

3)   STEVENS R, <u>HILAKIVI-CLARKE L</u>. Hypothesis: alcohol exposure *in utero* and later breast cancer risk. *Alcohol and Alcoholism* 36:2767, 2001. (In appendix A)

4)   <u>HILAKIVI-CLARKE LA</u>, FORSEN T, LUOTO R,ERIKSSON J, OSMOND C, BARKER D. Tallness and overweight during childhood have opposing effects on breast cancer risk. *Br. J Cancer*, in press 2001. (In appendix A)

5)   <u>HILAKIVI-CLARKE L</u>, CABANES A, OLIVO S, KERR L, BOUKER KB, CLARKE R. Do estrogens always increase breast cancer risk? *J Ster Biochem*, in press 2001. (In appendix A)

6)   JOHNSON M, KENNEY N, <u>HILAKIVI-CLARKE L,</u>, NEWBOLD R, CLARKE R, SHOLLER PF, LIRIO A, FOSS C, TROCK B, PAIK S, STOICA A, MARTIN MB. Cadmium mimics the effects of estrogen in vivo in the uterus and mammary gland. *Nature Med*, revision submitted 2001. (In appendix A)

7)   CABANES A, OLIVO S, DE ASSIS S, GUSTAFSSON J-A, <u>HILAKIVI-CLARKE L</u> Prepubertal estradiol exposure increases estrogen receptor beta levels in the mammary gland and reduces 7,12-dimethylbenz[a]-antracene-induced mammary tumorigenesis in rats. A manuscript in preparation, 2001.

8)   TROCK B, WHITE L, <u>HILAKIVI-CLARKE L</u>, CLARKE R. Meta-analysis of soy intake and breast cancer risk. A manuscript in preparation, 2001.

9)   <u>HILAKIVI-CLARKE L</u>, CHO E, CABANES A, DEASSIS S, OLIVO S, HELFERICH W, LIPPMAN ME, CLARKE R. Modulation of pregnancy estrogen levels by maternal dietary exposure to soy isolate or n-3 polyunsaturated fatty acid and breast cancer risk among female rat offspring. A manuscript in preparation, 2001.

10)   CABANES A, DEASSIS S, LIAO DJ, GUSTAFSSON J-A, HELFRICH W, HILAKIVI-CLARKE L. Soy diet during pregnancy reduces carcinogen-induced mammary tumorigenesis and causes a persistent increase in estrogen receptor beta protein levels in the rat mammary gland. A manuscript in preparation, 2001.

Key findings of the studies which have been completed and the manuscripts are in preparation are presented in Appendix B.

Task-2 . Identification of mechanistic pathways that mediate the effects of PUFA, genistein, and alcohol on breast cancer risk (months 12-36).

These intermediate biomarkers include:

2.1. Eicosanoids, particularly Cox-2

2.2. ER-α and ER-β

Research accomplished associated with Task-2

Findings concerning the mechanisms possibly mediating the effects of dietary manipulations on breast cancer risk are reported in the studies listed above (7, 9 and 10). Since all the manuscripts describing these results are still under preparation, key findings are presented in the appendix B.

Following published papers also address the plausible mediating mechanisms:

11)   HILAKIVI-CLARKE LA. Estrogens, BRCA1 and breast cancer. *Cancer Research*, 60:4993-5001, 2000. (In appendix A)

12)   BOUKER K, HILAKIVI-CLARKE LA. Genistein: A powerful weapon in the breast cancer prevention arsenal or a potent mitogen? *Environmental Health Perspectives* 108: 701-708, 2000. (In appendix A)

Eicosanoids

All appropriate mammary gland samples have been obtained from animals exposed to different dietary manipulations *in utero*, and we are planning to start to determine changes in COX-1 and COX-2 and PPAR expression during the last year of this academic award. Further, we are currently studying whether mammary tumorigenesis is affected in rats exposed to n-3 and n-6 PUFAs during prepuberty. Eicosanoid pathways will be determined from the glands obtained from the prepubertally manipulated animals also.

Alcohol

Studies to investigate the impact of timing of alcohol exposure on mammary tumorigenesis and the plausible mediating mechanisms will be performed during the last year of the Academic Award funding. The PI has published one paper this year on the connection between *in utero* alcohol exposure and breast cancer risk (see publication [3]). Further, the PI has submitted an idea grant proposal to 2001 DOD cycle to apply funding for a project studying the mechanisms mediating the effects of timing of alcohol exposure on the breast and is also an investigator in a 2001 DOD breast cancer center proposal which focuses on alcohol and the breast.

Task-3. Study whether (1) dietary fat intake and weight gain alter pregnancy estrogen levels in women, (2) affect possible intermediate biomarkers of increased breast cancer risk, as determined in nipple aspirate fluid, and (3) increase subsequent breast cancer risk (months 1-36)

3.1. The interactions among diet, weight gain and circulating estrogens in 200 pregnant women (months 1-18)
3.2. The effects of diet and weight gain on intermediate biomarkers in NAF (13-24)
3.3. Cohort study using >15,000 women to investigate whether pregnancy weight gain increases subsequent breast cancer risk. (24-36)

Research accomplished associated with Task-3

Results related to 3.1. The PI recently (funding begin 9/1/01) obtained an RO1 grant from the National Cancer Institute to expand the study which was initially funded by the Susan G. Komen Breast Cancer Foundation. The project will study associations between diet and serum hormone levels during pregnancy, and also explore the role of polymorphism in estrogen regulated genes in interacting with diet and pregnancy serum hormones. Further, we will include women to the study who are at high familial risk of developing breast cancer. For example, pregnancy always (regardless of age at first birth) increases breast cancer risk in BRCA1 mutation carriers. It is not known whether these women are particularly vulnerable to high pregnancy estrogen levels and whether the risk might not be elevated if estrogen levels remain at a lower range.

At present time we have recruited approximately 150 Swedish women to this study, and obtained blood samples from 100 of these women on gestation weeks 12, 25 and 32. These women have also completed three 24 hr dietary records on the same three gestation weeks. Biological assays to determine serum estrogen and growth factor levels during pregnancy will be performed in collaboration with Dr. Chantel Guillemette at the University of Laval in Quabec City, Canada. Dr. Guillemette has extensive experience in analyzing hormone levels in blood samples. Dr. Guillemette is able to start to analyze the samples in November.

Results related to 3.2. The PI and Co-Investigator (Dr. Bruce Trock) visited Karolinska Institutet in June to teach the nurse who is also collecting blood samples during pregnancy to perform nipple aspirate fluid sampling. The visit was very successful. Seven women with young children were invited to the clinic on the day of our visit. All but one of these women yielded NAF. We will begin to collect NAF from the women in our study in December 2001, which is 12 months after the first woman gave birth.

Results related to 3.3.

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We have completed a study to determine whether pregnancy weight gain affects mothers breast cancer risk, using a cohort consisting of 17,416 Finnish women. Dr. **Riitta Luoto**, MD/PhD, Senior Investigator at the University of Tampere, Finland, is the key collaborator in these studies. We are currently preparing a manuscript "HILAKIVI-CLARKE L, LUOTO R, HUTTUNEN T, KOSKENVUO M. Pregnancy weight gain and breast cancer risk - a case-control study" to describe the findings obtained in this study, and they can be summarized as follows:

Abstract

Background. High pregnancy estrogen levels have been associated with increased risk of developing maternal breast cancer. Since pregnancy weight gain may modify estrogen levels and animal studies indicate that high dietary fat intake both increases serum estrogens during pregnancy and mother's later breast cancer risk, we studied whether pregnancy weight gain increases breast cancer risk in women using a case-control design.

Material and Methods. Finnish women who had levonorgestrel-releasing intrauterine system (IUD) (MirenaR) inserted during 1990-93 composed the initial cohort. The physician inserting the IUD filled in a short demographic form for 26,630 women at the insertion visit. Another form was mailed in 1996 containing questions on overall health, and adult and pregnancy weight gain. This form was also given to sisters or female friends. 75% of the women responded to the second questionnaire. 114 of these women reported history of breast cancer, and 99 were available for the study. Four age- and Mirena- status matched controls for each case (N=396) were selected. Cox regression models were used in data analysis.

Results. As of 1996, mean age of the women who developed breast cancer and their controls was 46.6 years (range 32-58), and thus most of them were premenopausal. Women who developed breast cancer had higher level of education and age at first birth than the controls, consistent with other reports. The results also showed that higher education level was related to the higher age at first birth ($p < 0.0001$). Weight gain after age 20 (excluding pregnancy) was smaller among cases than controls. However, weight gain during pregnancy, when divided into four categories (<10 kg, 10-15 kg, 16-20 kg and >20 kg), did not affect breast cancer risk. The result did not change in the multivariate models which included all covariates.

Conclusion. Weight gain of over 20 kgs during pregnancy did not increase the risk of developing breast cancer in a cohort of mostly premenopausal Finnish women. However, since the highest weight gain category was only marginally higher than the recommended pregnancy weight, it remains to be determined whether a more excessive pregnancy weight gain affects mothers risk.

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Task-4. Development of Course in Nutrition and Breast Cancer (months 12-18)

4.1. Outline the course

4.2. Integration of the course to the existing Tumor Biology Course

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Research accomplished associated with Task-4.

(A) Attached is an outline of the course entitled "**Life-style and Cancer Prevention**" which has successfully been integrated to the existing tumor biology program and taught for the first time this spring semester.

(B) The PI was recently awarded by the National Cancer Institute a PO-type planning grant to set up a program to study interactions between diet, genes and cancer. The title of our funded proposal is "Timing of dietary exposures and breast cancer risk: role of steroid receptors and tumor suppressor genes". The planning grant is anticipated to lead to a successful competition of U54 grant, which consists of several RO1 type projects. Thus, this U54 program is partly comparable to SPORE funding mechanism.

KEY RESEARCH ACCOMPLISHMENTS

Bulleted list of key research accomplishments:

- Dietary modulations occurring during pregnancy can alter both **rat** dam's and her female offspring's breast cancer risk.

   Mother
   > Dietary factors which increase mother's breast cancer risk:
   >> high fat n-6 polyunsaturated fatty acids

   > Dietary factors which reduce offspring's breast cancer risk:
   >> genistein in soy isolate feed

   Offspring
   > Dietary factors which increase offspring's breast cancer risk:
   >> high fat n-6 polyunsaturated fatty acids
   >> genistein (administered subcutaneously)

   > Dietary factors which do not affect offspring's breast cancer risk:
   >> genistein in soy isolate feed

Dietary factors which reduce offspring's breast cancer risk:
high fat n-3 polyunsaturated fatty acids.

- Mechanisms possibly mediating the effects of *in utero* dietary modifications:

  - increased pregnancy estradiol levels
  - increased number of cellular targets for malignant transformation
  - increased expression of ER-alpha protein and reduced expression of ER-beta protein.

- Dietary modulations before puberty may alter later breast cancer risk.

  Animal data:
  prepubertal genistein or estradiol exposure reduces the risk.

  Human data:
  high body mass during childhood is associated with reduced breast cancer risk.

- Mechanisms mediating the effects of prepubertal dietary modifications:

  - increased childhood estrogen exposure
  - reduced number of cellular targets for malignant transformation via elimination of undifferentiated epithelial structures
  - reduced expression of ER-alpha protein and increased expression of ER-beta protein.
  - increased expression of BRCA1

- Translational studies in human populations have been initiated to determine whether findings obtained in animal studies are true also for women.

  "Karolinska study": Interactions among pregnancy diet, estrogen levels and intermediate biomarkers of increased breast cancer risk determined in nipple aspirate fluid. - ongoing

  "Finnish study 1": Growth during childhood and later breast cancer risk. - completed

  "Finnish study 2": Pregnancy weight gain and mother's later breast cancer risk. - one study completed, other ongoing

  "Finnish study 3": Impact of body weight at birth, during childhood and pregnancy on the penetrance of breast cancer in women at high familial breast cancer risk. - in planning phase

- Course addressing the role of life-style, including diet, in affecting cancer risk put

together and taught for graduate students during spring 2001.

- Funding (planning grant) obtained from the National Cancer Institute to create a novel program on nutrition, genes and cancer.

REPORTABLE OUTCOMES (7/1/00-8/31/01):

Manuscripts

HILAKIVI-CLARKE LA. Estrogens, BRCA1 and breast cancer. *Cancer Research*, 60:4993-5001, 2000.

CABANES A, DE ASSIS S, GUSTAFSSON J-A, HILAKIVI-CLARKE L, . Maternal high-fat intake during pregnancy increases voluntary alcohol intake and hypothalamic estrogen receptor protein levels among female offspring. *Dev Neuroscience*, in press 2000.

BOUKER K, HILAKIVI-CLARKE LA. Genistein: A powerful weapon in the breast cancer prevention arsenal or a potent mitogen? *Environmental Health Perspectives* 108: 701-708, 2000.

HILAKIVI-CLARKE L, CHO E, DEASSIS S, OLIVO S, EALLEY E, BOUKER KB, WELCH J, KHAN G, CLARKE R, CABANES A. Maternal and prepubertal diet, mammary development, and breast cancer risk. *J Nutr* 131:154S-157S, 2001.

CLARKE R, HILAKIVI-CLARKE LA, TROCK B. Dietary and environmental sources of estrogenicity and breast cancer risk. *Biologist* 48:21-6, 2001.

STEVENS R, HILAKIVI-CLARKE L. Hypothesis: alcohol exposure *in utero* and later breast cancer risk. *Alcohol and Alcoholism* 36:276-7, 2001.

HILAKIVI-CLARKE LA, FORSEN T, LUOTO R,ERIKSSON J, OSMOND C, BARKER D. Tallness and overweight during childhood have opposing effects on breast cancer risk. *Br J Cancer*, in press 2001.

CABANES A, OLIVO S, DE ASSIS S, GUSTAFSSON J-A, HILAKIVI-CLARKE L Prepubertal estradiol exposure increases estrogen receptor beta levels in the mammary gland and reduces 7,12-dimethylbenz[a]-antracene-induced mammary tumorigenesis in rats. Submitted, 2001.

HILAKIVI-CLARKE L, CABANES A, OLIVO S, KERR L, BOUKER KB, CLARKE R. Do estrogens always increase breast cancer risk. *J Steroid Biochem*, in press 2001.

JOHNSON M, KENNEY N, HILAKIVI-CLARKE L,, NEWBOLD R, CLARKE R, SHOLLER PF, LIRIO A, FOSS C, TROCK B, PAIK S, STOICA A, MARTIN MB. Cadmium mimics the

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effects of estrogen in vivo in the uterus and mammary gland. *Nature Med*, revision submitted 2001.

Manuscripts in preparation
TROCK B, WHITE L, HILAKIVI-CLARKE L, CLARKE R. Meta-analysis of soy intake and breast cancer risk. A manuscript in preparation, 2001.

HILAKIVI-CLARKE L, LUOTO R, HUTTUNEN T, KOSKENVUO M. Pregnancy weight gain and breast cancer risk - a case-control study. A manuscript in preparation, 2001.

HILAKIVI-CLARKE L, CHO E, CABANES A, DEASSIS S, OLIVO S, HELFERICH W, LIPPMAN ME, CLARKE R. Modulation of pregnancy estrogen levels by maternal dietary exposure to soy isolate or n-3 polyunsaturated fatty acid and breast cancer risk among female rat offspring. A manuscript in preparation, 2001.

CABANES A, DEASSIS S, LIAO DJ, GUSTAFSSON J-A, HELFRICH W, HILAKIVI-CLARKE L. Soy diet during pregnancy reduces carcinogen-induced mammary tumorigenesis and causes a persistent increase in estrogen receptor β protein levels in the rat mammary gland. A manuscript in preparation, 2001.

Presentations

2000	Annual Conference of American Institute for Cancer Research. A symposium presentation in the session "Nutrition, Normal Development and Cancer Prevention" entitled *"Maternal and prepubertal diet, mammary development and breast cancer risk"*. In Washington DC, September 2000.
2000	The Susan G. Komen *Reaching for the Cure ... Making A Difference* Mission Conference. Oral presentation entitled *"Timing of estrogen exposure and breast cancer risk"*. In Washington DC, September 2000.
2000	The Third Cooper Institute Scientific Conference: Physical Activity and Cancer. A symposium presentation entitled *"Physical activity, stress adaptation - habituation, and cancer"*. In Dallas, Texas. November 5-7, 2000.
2001	5th Annual Breast Cancer Symposium by the Expedition Inspiration Fund for Breast Cancer Research, entitled "Hormones and Breast Cancer: Pathogenesis, Prevention, Treatment."
2001	6th Annual Oncology Conference, University of Missouri. Keynote presentation, entitled *" Timing of genistein exposure and breast cancer risk."* Missouri, April 27.
2001	Gordon Conference entitled "Hormonal carcinogenesis", presentation in

the late breaking research session entitled *"Dietary factors in hormonal carcinogenesis."* Kimball Union Academy, July 8-13, 2001.

Funding

I . <u>Current support</u>

Research Grant (PI: Hilakivi-Clarke)   3/1/99-3/1/2002

Susan G. Komen Breast Cancer Foundation $96,800 (first year)

"Pregnancy estrogens, diet, and intermediate biomarkers of breast cancer risk in nipple aspirate fluid"

> This grant have provided seed funding to investigate the role of diet in affecting pregnancy estrogens, and their possible connection to biomarkers of increased breast cancer risk. At present time, 100 women have been recruited to the project, and we have obtained blood samples from these women 2-3 times during their pregnancy. These women will start to give birth this summer. <u>None of the blood samples or dietary intakes have been analysed</u>. Because of the 1RO1CA89950 award, we have rebudgeted the Komen grant for 2 other epidemiological studies that will be done in collaboration with Dr. Riitta Luoto, University of Tampere, Finland. These studies are investigating whether pregnancy weight gain correlates with breast cancer risk using existing data in two different cohorts. Thus, there will be <u>no</u> <u>overlap between the existing Komen grant and the 1RO1CA89950 award</u>.

Academic Award (PI: Hilakivi-Clarke)   7/1/99-7/1/2002

US Army Medical Research and Material Command $77,400 (first year)

"Dietary prevention of breast cancer"

> This is an Academy Award type of a grant that provides 100% salary support to appraise critically the state of dietary prevention of breast cancer.

RO1 (PI: Hilakivi-Clarke)   7/1/01-6/30/05

National Cancer Institute $1,000,000 (total, Modular grant)

"Pregnancy estrogens, diet, and breast cancer risk"

> This grant will provide funding to a project to be conducted in collaboration with Karolinska Institutet in Sweden to study factors during pregnancy that might influence breast cancer risk in mothers.

Research Grant (PI: Leena Hilakivi-Clarke)   10/1/01-10/1/03

Breast Cancer Research Foundation $250,000 (total)

"Early life exposure to whole grains and breast cancer risk"

> This project investigates whether some breast cancers can be prevented by dietary exposure to fiber, either during fetal life through pregnant mother or during childhood.

Postdoctoral Fellowship (PI: Leena Hilakivi-Clarke, fellow: Anna Cabanes)9/15/01-9/15/03

Cancer Research Foundation of America

"Soy and breast cancer"

This fellowship will support Dr. Anna Cabanes's research studying the role of ERbeta in the mammary gland.

Research Grant (PI: Kim Westerlind)   9/15/01-9/15/02

Cancer Research Foundation of America

"Prepubertal physical activity and rat mammary tumorigenesis"

This project will study whether prepubertal physical activity affects mammary tumorigenesis and the mechanisms mediating the association.

RFA: CA-01-015 (PI: Leena Hilakivi-Clarke)

National Cancer Institute

"Timing of dietary exposures and breast cancer risk: role of steroid receptors and tumor suppressor genes".

Planning Grant for collaborations on nutritional modulation of genetic pathways leading to cancer. Total award amount: $74,555 for 6 months.

Research Grant (PI: Hilakivi-Clarke)   1/1/02-1/1/04

Susan G. Komen Breast Cancer Foundation   Total award amount: $249,880 for 2
                                    years.

"Timing of estrogen exposure and breast cancer risk".

This grant will provide funding to investigate the biological mediators of *in utero* and prepubertal estrogenicity on breast cancer risk.

II. Pending support

Idea Grant (PI: Hilakivi-Clarke)   7/1/02-7/1/06

US Army Medical Research and Material Command $6,858,476 (total costs)

"Alcohol and Increased Breast Cancer Risk: Role of ER(alpha), ER(beta) and BRCA1"

This project will investigate the mediators linking alcohol intake to increased breast cancer risk.

Breast Cancer Center of Excellence (PI: Shields)   7/1/02-7/1/05

US Army Medical Research and Material Command

"Why does alcohol drinking cause breast cancer"

The purpose of this grant application is to create a DOD funded Breast Cancer Center

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focusing on alcohol research which also has technical capabilities to transmit information and research data between centers.

CONCLUSIONS:

Summary of the results

The data obtained during the first two years of funding emphasize that estrogens have different effects on breast cancer risk, depending on the timing of exposure. Some dietary components have estrogenic effects. Our studies have shown that a maternal exposure to a high n-6 or n-3 PUFA diet increases pregnancy estrogen levels. Phytoestrogens, including genistein present in soy, appear to activate the estrogen receptor. We found that *in utero* exposure to some dietary components (high fat n-6 PUFA, genistein) which either increase pregnancy estrogen levels or activate the estrogen receptor increase offsprings' mammary tumorigenesis, while some have no effect (genistein in soy isolate) and some (high fat n-3 PUFA) reduce the risk. These results suggest that soy isolate and n-3 PUFA contain components which can counteract the effect of high *in utero* estrogen levels on breast cancer risk. Studies are currently in progress in human populations to determine whether (1) diet during pregnancy affects pregnancy estrogen levels and intermediate biomarkers of elevated breast cancer risk in nipple aspirate fluid; (2) pregnancy weight gain affects later breast cancer risk; and (3) indicators of high pregnancy estrogen levels affect penetrance of familial breast cancer.

Prepubertal exposure to estrogenic exposures paradoxically reduce later breast cancer risk. Our results obtained in animal models suggest that both prepubertal exposure to estradiol or genistein reduces the likelihood that rats will develop carcinogen-induced mammary tumorigenesis. Data obtained in a cohort study support the protective effect of high childhood estrogenic environment and show that high body mass during childhood is linked to reduced breast cancer risk.

The results strongly suggest that a new variable, timing of exposure should also be taken into account when the effects of diet on the risk of developing breast cancer are being assessed.

Our data further indicate that the mechanisms by which early life dietary exposures affect breast cancer risk are related to changes in the mammary gland differentiation and expression of ER-α and ER-β. In particular, increase in ER-β protein and BRCA1 levels might protect the breast from malignant transformation. During the last remaining year, we will be doing further studies to determine the specific roles of the two estrogen receptors and tumor suppressor genes as well as eicosanoids in affecting the risk of developing breast cancer. We also will attempt to identify the best means to prevent some breast cancer by dietary modifications during childhood and pregnancy.

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So what section

Although diet is clearly associated with breast cancer risk, studies have failed to provide convincing evidence in favor of a particular dietary component in causing or preventing breast cancer. This failure is likely to have been caused by not having been taking into consideration the fact that timing of exposure is critical. An exposure to the same dietary component might have a different effect on breast cancer risk, if the exposure occurs *in utero* through a pregnant mother, during childhood, puberty, pregnancy, reproductive years or postmenopause. For example, our recent results and the results of other investigators indicate that an exposure to a phytoestrogen genistein during fetal life and postmenopause might increase breast cancer risk, while an exposure during childhood may provide a permanent protection. By determining the impact of timing of various dietary components, we are more likely to be able to prevent some breast cancers than by assessing the interaction between diet shortly before diagnosis and breast cancer risk.

APPENDICES

Appendix A. COPIES OF PUBLISHED, ETC. WORK

Maternal and Prepubertal Diet, Mammary Development and Breast Cancer Risk[1,2]

L. Hilakivi-Clarke,[3] E. Cho, S. deAssis, S. Olivo, E. Ealley, K. B. Bouker, J. N. Welch, G. Khan, R. Clarke and A. Cabanes

Department of Oncology, Lombardi Cancer Center, Georgetown University, Washington, DC 20007

Although diet has been implicated as playing a major role in breast cancer, we do not know what dietary factors are responsible for initiating and promoting breast cancer. Until recently, a high intake of dietary fat was believed to contribute to the high incidence of breast cancer in the Western world. However, results obtained in cohort studies indicate that this may not be the case (Hunter et al. 1996). Another dietary component that has been linked to breast cancer is soy. High soy intake has been suggested to lower breast cancer incidence in Asian countries (Adlercreutz et al. 1996). However, a meta-analysis of results obtained in case-control and cohort studies indicates that high soy intake does not reduce cancer risk, at least in postmenopausal women (Trock et al. 2000). A consensus exists that high vegetable intake reduces breast cancer risk, but none of the suspected components in vegetables appear to explain their protective effect. Thus, the dilemma is that although diet is estimated to contribute to at least 50% of newly diagnosed breast cancers, the specific dietary components associated with breast cancer etiology remain unknown.

Estrogen exposure and breast cancer risk

One factor contributing to the current confusion regarding diet and breast cancer is that the same dietary component might have a different—even opposing—effect on breast cancer risk, depending on the timing of exposure. In the case of estrogens, where the evidence strongly indicates that they increase breast cancer risk, timing of exposure is important. Estrogens stimulate the growth of human breast cancer cells in vitro (Dickson and Russo 2000), and estrogen exposure increases breast cancer risk, at least in postmenopausal women (Hankinson et al. 1998). Further, several reproductive factors

indicating an increased exposure to estrogens also increase breast cancer risk (Hulka and Stark 1995), whereas reduction in ovarian estrogen levels by bilateral ovariectomy markedly reduces breast cancer risk (Kreiger et al. 1999). However, circulating estrogen levels during the reproductive years are not associated with a risk of developing premenopausal breast cancer (Key et al. 1996). A modest reduction in circulating estrogens, such as that produced by unilateral ovariectomy (Parazzini et al. 1997), or oral contraceptive (Romieu et al. 1990) or contraceptive depot use (Paul et al. 1989) (both inhibit ovulation and ovarian estrogen production) actually increases rather than reduces breast cancer risk. Further, a 10-fold increase in circulating estrogens during pregnancy (Yuan et al. 1988), a short menstrual cycle length (increased exposure to ovarian estrogens) (Titus-Ernstoff et al. 1998) or a high body mass index (BMI)[4] (increased exposure to adipose tissue–derived estrogens) reduces the risk for developing premenopausal breast cancer.

In utero estrogen exposure and breast cancer risk

It has been suggested that the higher the in utero estrogenicity, the greater the subsequent risk of developing breast cancer (Trichopoulos 1990). This hypothesis is supported by data obtained in epidemiologic studies (Ekbom et al. 1992, Michels et al. 1996, Weiss et al. 1997). Animal studies indicate that a maternal exposure to an elevated estrogenic environment, as induced by an administration of either estradiol (Hilakivi-Clarke et al. 1997b) or the synthetic estrogen diethylstilbestrol (DES) (Walker 1984), significantly increases breast cancer risk in female offspring.

The concept that a high in utero estrogenicity increases breast cancer risk has been challenged recently by clinical findings showing that circulating estrogen levels are significantly higher in pregnant Asian women, who exhibit low breast cancer risk, than in Caucasian women, who exhibit high breast cancer risk (Lipworth et al. 1999). Because nonpregnant Asian women have ~40% lower serum estrogen levels than do Caucasian women (Goldin et al. 1986), it is

[4] Abbreviations used: BMI, body mass index; DES, diethylstilbestrol; DMBA, 7,12-dimethylbenz[a]anthracene; EGF, epidermal growth factor; ER, estrogen receptor; LAU, lobuloalveolar units; PUFA, polyunsaturated fatty acids; TEB, terminal end buds; TGF-α, transforming growth factor α.

essential to determine why the reverse occurs during pregnancy. Our animal studies (Cabanes et al., unpublished data) suggest that soy and fish oil, both typical to Asian diets, increase circulating estrogen levels during pregnancy, whereas they have an opposite effect on offspring (i.e., adult female offspring of dams who consumed a high soy diet during pregnancy exhibit reduced estrogen levels).

Maternal diet and offspring's breast cancer risk

We have studied the effect of maternal diets on mammary tumorigenesis in female rat offspring. The diets were those with high or low amounts of (n-6) polyunsaturated fatty acids (PUFA) (Hilakivi-Clarke et al.1997b), genistein (Hilakivi-Clarke et al. 1999) or soy (Cabanes et al., unpublished data). The source of the (n-6) PUFA was corn oil, and it significantly increased circulating estradiol levels in pregnant rats (Hilakivi-Clarke et al. 1996 and 1997b). Genistein is a phytoestrogen and the component believed to mediate soy's effects on the breast; however, it binds and activates the estrogen receptor (ER) and therefore stimulates the growth of normal and malignant mammary cells both in vitro and in vivo (Bouker and Hilakivi-Clarke 2000).

The results indicate that high amounts of (n-6) PUFA and genistein both increase the offspring's mammary tumorigenesis if exposed through a pregnant rat dam (Hilakivi-Clarke et al. 1997b and 1999). The carcinogen used to induce tumors was 7,12-dimethylbenz[a]anthracene (DMBA) administered to offspring as a single oral 10-mg dose. Maternal soy intake did not increase offspring's risk of developing mammary tumors (Cabanes et al., unpublished data), although the soy diet contained high levels of genistein. The soy diet also increased pregnancy estrogen levels. These results indicate that soy must contain some additional components, which reverse the effects of genistein on offspring's breast cancer risk when administered in utero.

Prepubertal estrogen exposure

Little is known about estrogenicity during childhood and its effect on later breast cancer risk. In a unique study with rats, daily exposure to estradiol between postnatal d 0 and 30 effectively reduced susceptibility to carcinogen-induced mammary tumorigenesis (Nagasawa et al. 1974). However, alterations in reproductive parameters were also noted, which could potentially affect fertility. We recently found that a shorter exposure (between postnatal d 7 and 20) at lower estradiol concentrations also reduced carcinogen-induced mammary tumorigenesis in rats without causing alterations in the reproductive system (Cabanes et al., unpublished data).

In humans, the effect of childhood exposure to estrogens has been studied indirectly by investigating associations among BMI, height and dietary fat intake. Obese girls have more adipose tissue than thin girls, and adipose tissue is the site of the conversion of adrenal androgens to estrogens. Four recent studies, including our cohort study, indicate that women who had a high BMI between ages 7 and 15 y exhibit significantly reduced breast cancer risk (Berkey et al. 1999, Hilakivi-Clarke et al. 2000, Le Marchand et al. 1998, Magnusson et al. 1998). Breast cancer risk is also reported either not to be affected or reduced in women who consumed a high fat diet around the time of puberty (Hislop et al. 1986, Potischman et al. 1998). Height in childhood is linked directly with later breast cancer risk; that is, if a girl is tall at age 7 y, her risk of developing breast cancer is significantly increased (Hilakivi-Clarke et al. 2000). Childhood height is inversely linked to

circulating estrogen levels (Jacklin et al. 1994). These findings support the data obtained in animal studies indicating that an exposure to estrogens in childhood reduces later breast cancer risk.

Prepubertal diet and breast cancer risk

As indicated above, human studies suggest that high fat intake at puberty does not increase breast cancer risk but rather reduces it. To our knowledge, no animal studies exist that have examined the effects of an exposure to dietary fat before weaning on mammary tumorigenesis. We are currently performing such a study in rats that were exposed to high (n-6) PUFA diet between postnatal d 7 and 21.

Our group (Hilakivi-Clarke et al. 1998) and the group of Dr. Lamartiniere (Murrill et al. 1996) have been studying the effects of prepubertal exposure to genistein on DMBA-induced mammary tumorigenesis. We (Hilakivi-Clarke et al. 1998) also have studied the effects of prepubertal exposure to another phytoestrogen, zearalenone—a contaminant in grains, corn, potato, rice and other similar farm products that effectively activates the ER. Exposure to either of these two phytoestrogens between postnatal d 7 and 20 effectively reduced carcinogen-induced mammary tumor incidence.

Mechanisms mediating in utero estrogenic exposures of the breast

Mammary gland morphology. Estrogenic exposures in early life play a central role in the development of the normal mammary gland. The effects in rodents and humans appear markedly similar, perhaps reflecting the many structural, functional and endocrinologic similarities between the mammary glands in these species (Russo et al. 1990). In rodents (as well as in humans), the rudimentary mammary gland develops in utero and is characterized by growth of the primary branch from the nipple with subsequent limited secondary branching. This process depends on transplacental maternal hormones of pregnancy, including estrogens (Dickson and Russo 2000).

We have found that in utero exposure to estradiol, high (n-6) PUFA or genistein alters normal mammary gland development. The glands exposed to estrogenic compounds in utero contain persistent terminal end buds (TEB), exhibit reduced differentiation to lobuloalveolar units (LAU) or both (Hilakivi-Clarke et al. 1997a and 1997b). TEB play a central role in mammary gland development. They are the most actively growing epithelial structures and contain cap cells that are interpreted to represent a pluripotent stem cell population (Russo and Russo 1996). These cap cells are located on the basal surface of the TEB beneath the basal lamina. The TEB are known to be the sites of malignant transformation in the rodent mammary gland and possibly also in the human breast (Russo and Russo 1996). These data clearly support the hypothesis that perinatal exposure to estrogenic compounds can alter mammary gland development, which in turn might be associated with increased susceptibility to develop breast cancer.

Epigenetic mechanisms. The ER is a likely mediator of the effects of in utero estrogenic exposures on mammary tumorigenesis. Two ER subtypes have been identified in the mammary gland, i.e., the classical ER-α and a novel ER-β (Kuiper et al. 1996). ER-α is known to be associated with increased cell proliferation and breast cancer risk, but it is not clear whether activation of ER-β has similar or different effects on cells than activation of ER-α. Some evidence suggests that activation of ER-β may prevent epithelial cell proliferation,

including the proliferative effects resulting from activation of ER-α, and thus protect the gland (Paech et al. 1997, Saji et al. 2000). Further, ER-β levels are highest in normal breast tissue and lowest in malignant tumors (Leygue et al. 1998), suggesting that low ER-β levels are associated with increased risk to develop breast cancer.

Earlier studies measured ER levels in the mammary glands of rodents exposed to estrogenic manipulations in utero using a ligand binding assay that determines total ER binding (including both ER-α and ER-β). The data indicate that a maternal exposure to DES reduces total ER binding sites in the offspring's mammary gland or tumors (Bern et al. 1985, Verhoeven et al. 1982). Because ER-β levels are higher than ER-α levels in the virgin rat mammary gland (Saji et al. 2000), the decrease in total ER binding sites after DES exposure may reflect a reduction in ER-β levels. This would allow ER-α to induce cell proliferation without being opposed by ER-β. In support of this view, our preliminary data obtained in female rat offspring whose mothers were fed a diet high in (n-6) PUFA during pregnancy indicate that their mammary glands contained increased levels of ER-α protein and reduced levels of ER-β protein (Cabanes et al., unpublished data). Further, it has been noted that neonatal estrogenization of male rats, which increases susceptibility to estrogen-induced carcinogenesis of the urogenital tract, leads to increased expression of ER-α but decreased expression of ER-β in the adult prostate (Prins et al. 1998).

We have found that a maternal exposure to genistein, in contrast, increases mammary ER binding sites in the offspring (Hilakivi-Clarke et al. 1999). This increase may reflect upregulation of ER-α, again causing an increase in unopposed cell proliferation. The latter interpretation is supported by the observation that genistein preferentially binds to (and perhaps down-regulates) ER-β (Kuiper et al. 1997) and thus may up-regulate ER-α as a consequence of in utero exposure. Because in utero exposure to a high fat diet, DES or genistein increases carcinogen-induced mammary tumorigenesis, changes in either ER-α or ER-β might be critical in terms of determining the susceptibility to breast cancer.

Mechanisms mediating prepubertal estrogen exposures on the breast

Mammary gland morphology. During puberty, ductal elongation and branching occur in the mammary gland, and ovarian estrogens participate in regulating this process. In particular, estrogens induce the growth of mammary ducts, and estrogen-induced progesterone induces lobuloalveolar growth (Russo and Russo 1996). There is some dispute concerning whether estrogens can directly mediate mitogenic effects on the mammary gland or whether they occur through estrogen-induced stimulation of growth factors, such as epidermal growth factor (EGF) and transforming growth factor α (TGF-α) (Dickson and Russo 2000). In normal mammary epithelial cells, the ER is not located in cells that proliferate but in cells immediately next to them. Therefore, the proliferation of normal mammary epithelial cells that are exposed to estrogens probably occurs via estrogen-induced stimulation of, for example, TGF-α, which is located in the TEB of proliferating cells (Snedeker et al. 1991).

We (Hilakivi-Clarke et al. 1998) and others (Murrill et al. 1996) have noted that prepubertal exposure to genistein causes changes in the mammary gland that become apparent a few weeks after the cessation of prepubertal exposure. These changes can be characterized as increased differentiation of TEB to LAU. The differentiated mammary gland exhibits low

or no susceptibility to carcinogen-induced malignancies, whereas nondifferentiated gland containing high levels of TEB is particularly prone to develop malignancies if exposed to carcinogens (Russo et al. 1990).

Epigenetic mechanisms. A complex interplay among various hormones, growth factors and other pathways is likely to be responsible for the differentiation of the mammary epithelial tree. Lamartiniere's group (Brown et al. 1998) showed that prepubertal exposure to genistein modulates expression of estrogen-regulated growth factors such as TGF-α, EGF and EGF receptor in the mammary gland. We have found that ER-α and ER-β protein levels are altered in the mammary glands of rats exposed to estradiol during the prepubertal period (Cabanes et al., unpublished data). Specifically, loss of ER-α protein expression and an almost fourfold increase in ER-β protein levels occur in the mammary glands of rats exposed prepubertally to estradiol. These findings suggest that a reduction in ER-α and estrogen-mediated growth factor pathways is linked to reduced mammary tumorigenesis. Further, an increase in ER-β protein levels in the mammary gland of rats exposed to estradiol during prepuberty may be associated with reducing their risk for developing mammary tumors.

SUMMARY

At present, we do not know what causes sporadic breast cancer. Environmental factors, particularly diet, appear to explain at least 70% of newly diagnosed breast cancers, but it is not clear what these factors are. We propose that the lack of progress in this area is due to a lack of considering the effect of timing of environmental and dietary exposures on the breast. The evidence provided above suggests that an in utero exposure to an estrogenic environment—including that caused by diet [high (n-6) PUFA or genistein]—increases breast cancer risk. This increase may be mediated by an increased presence of TEB in the mammary epithelial tree and increased ER-α levels, reduced ER-β levels or both. Prepubertal estrogenic exposure, in contrast, reduces later risk of developing breast cancer. The protective effect of estrogens may be mediated by early epithelial differentiation, reduced presence of ER-α and increased levels of ER-β in the mammary gland. The challenge we are now facing is to determine whether the data obtained mainly through the use of animal models is relevant to women and if so, how we might be able to modulate pregnancy and childhood estrogenic exposure by appropriate dietary modifications to reduce breast cancer risk in women.

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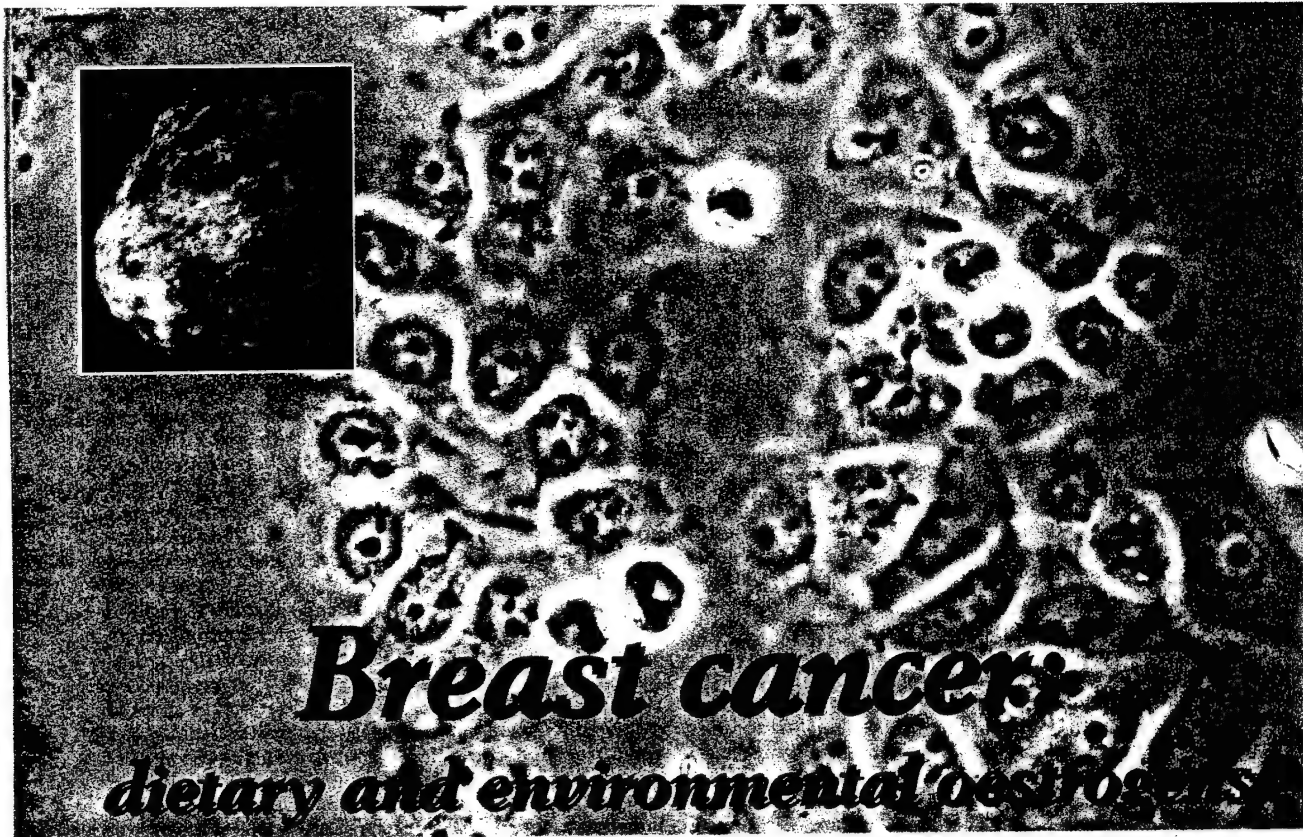
MCF-7 human breast cancer cells growing in vitro. Mammogram inset.

Breast cancer: dietary and environmental oestrogens

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Breast cancer is among the most common of the cancers that occur in women living in western societies. It is a much-feared disease and the risks are confusing and often badly reported. Oestrogen levels are a known risk factor. But is it possible to lower the risk? And where are all the oestrogens coming from?

The incidence of breast cancer is high among women in western societies. The disease has a high mortality rate when local treatment (surgery and radiotherapy) does not produce a cure. A major problem for oncologists is the management of the cancer once it has spread beyond the breast (metastatic disease). Many treatments, with the possible exception of hormone-based therapies, are toxic and often produce severe side effects. The most effective approaches to eradicating this disease may come from the area of prevention. To identify effective preventive treatments, it is important to determine the cause(s) of the disease, or at least identify controllable factors that can reduce disease risk. While several risk factors are known, most cannot easily be influenced. To complicate things further, many patients present with few, if any, of the known risk factors.

More than 200 years ago, the Italian physician Ramazzini observed an increased incidence of breast cancer among nuns. It is now well established that never having had children is associated with an increased breast cancer risk. Conversely, risk is reduced if a woman has her first full term pregnancy at a young age (<20 years) and further reduced by extended breastfeeding and multiple pregnancies. One hundred years ago the Scottish physician Beatson described the beneficial effects of removing a woman's ovaries on the progress of breast cancer in premenopausal women. This remains an effective treatment but has been largely replaced by either the use of drugs that block oestrogen biosynthesis or action, or by destruction of the ovaries by irradiation. Breast cancer risk is increased both in women who begin menstruating at a young age (<12 years) and in women who enter the menopause at an older age (Hulka and Stark, 1995). Together, these observations show a clear case for ovarian oestrogens in breast cancer risk.

If the case for internal oestrogens is well established, what then of external oestrogens? This area is controversial, often simply because the data are sparse and contradictory. Nonetheless, oestrogenicity is pervasive in our environment and has been associated with several adverse effects. Plant chemicals with oestrogenic activities (phyto-oestrogens) are responsible for diseases such as clover disease, which causes severe infertility in sheep. Contamination of alligator habitats with man-made oestrogenic chemicals (xeno-oestrogens) affects the sexual development of alligators.

The two primary classes of oestrogenic compounds in our environment are phyto- and xeno-oestrogens. The first class is natural: phytochemicals in plants and plant products, which probably provide some benefit to the plants in which they occur. (It has been suggested that they help protect plants by interfering with the reproductive cycle or

development of parasitic insects.) The second class of compounds (called either environmental oestrogens, or xeno-oestrogens) includes pesticides, industrial waste products and other man-made chemicals and pollutants.

Oestrogenicity and oestrogen receptors

Oestrogens are ligands for (or compounds that bind to) two nuclear hormone receptors, the alpha (ERα) and beta (ERβ) oestrogen receptors. These are the products of two different genes. In the cancerous breast, ERα expression may predominate. It is likely that most of the oestrogenic effects in the mature gland are mediated by ERα. The role of ERβ is less well understood, the gene being cloned relatively recently. ERs are nuclear transcription factors, that is they regulate the expression of other genes. They do so by binding to specific DNA sequences called oestrogen responsive elements (EREs). Coregulator proteins are also recruited and affect the transcription of the adjacent gene (Figure 1). There are many oestrogen-regulated genes, including the progesterone receptor, the protease cathepsin D, the epidermal growth factor receptor and several growth factors that stimulate cell proliferation (mitogens). The precise oestrogen-regulated genes that drive the proliferation of normal and neoplastic breast tissues are not known.

Phyto-oestrogens

If we define an oestrogen in terms of its ability to bind and activate ER and, consequently, regulate gene expression, then we also can loosely apply this simplistic definition to phytochemicals. Thus, a phyto-oestrogen could be any plant-derived compound that is capable of activating ER (Clarke *et al.*, 1996). From the perspective of breast cancer research, there are probably 'good' phyto-oestrogens and 'bad' phyto-oestrogens. The timing and dose of exposure may be crucial, meaning that the same compound is 'good' in some circumstances and 'bad' in others. Phyto-oestrogens can also have other properties. For example, genistein (found in soy and some soy products) is a phyto-oestrogen and an inhibitor of both topoisomerase II and some tyrosine kinases. In many cases it may be difficult to distinguish between these mechanisms, particularly when an oestrogenic pathway includes the regulation of tyrosine kinase activities.

Where do you find phyto-oestrogens and what is the level of exposure?

There are several groups of plant oestrogens. These include the lignans such as enterolactone, isoflavones such as genistein, and fungal mycotoxins such as zearalenone (see Table 1 and Figure 2). Lignans and isoflavones are found in whole grain and soy products, fruits and berries (Table 1). Levels of exposure to these phyto-oestrogens can be high and they are a major source of oestrogenicity in prepubertal and postmenopausal women. Other sources come from the conversion of adrenal androgens (male sex hormones) to oestrogens in peripheral adipose (fat) tissues and, for some postmenopausal women, hormone replacement therapy.

In soy products the major oestrogenic phyto-oestrogens are the isoflavones genistein and daidzein. They are actually present as the glycosides (sugar containing) genistin and daidzin but the sugar group is removed by gut microflora, leaving genistein and daidzein. They are then absorbed into the circulation. Genistein exposure is approximately 1.5–4.1 mg/person (~0.05–0.1 mg/kg) in Asia, and approximately 20 times less (at most ~0.05 μg/kg) in the US and EU. These levels reflect the marked dietary differences among these populations. It has been suggested that the difference in soy consumption may contribute to the lower incidence of breast cancer in Oriental countries. However, this is probably a simplistic interpretation.

Zearalenone has also begun to attract attention. Zearalenone is mainly produced by the mould *Fusarium graminearum*. It is found in a variety of host plants and soil debris. It is present as a contaminant in stored cereals, *e.g.*, barley, wheat, corn, corn flakes, and rice, at concentrations from 35–115 g/kg. Individuals living in the US are exposed to 1–5 mg/day (0.02–0.1 mg/kg/day) of zearalenone, a level comparable to the exposure to genistein in the East.

Some alcoholic beverages contain phyto-oestrogens. Bourbon contains the phyto-oestrogens biochanin A and β-sitosterol, whereas beer contains genistein. In grapes and wines the potentially active compound is resveratrol, which is believed to act as a natural antifungal. Consumption of wines is associated with reduced risk of cardiovascular disease. Since oestrogens have protective effects in this context, these could be partly produced by the oestrogenicity in some alcoholic beverages. Generally, alcohol consumption is associated with an increased risk of developing breast cancer. The precise mechanism is unclear but alcohol is known to increase serum oestrogen levels, in addition to the oestrogenic activity of any phyto-oestrogens present.

Indole-3-carbinol is found in *Brassica* species, such as broccoli, and has been identified as a weak phyto-oestrogen. Its most important activity may be to alter the metabolism of the natural oestrogens to less chemically reactive oestrogen metabolites (Telang *et al.*,1997).

The lignans enterolactone and enterodiol are formed by the action of gut microflora on precursors present in grains, seeds, berries and nuts. Women
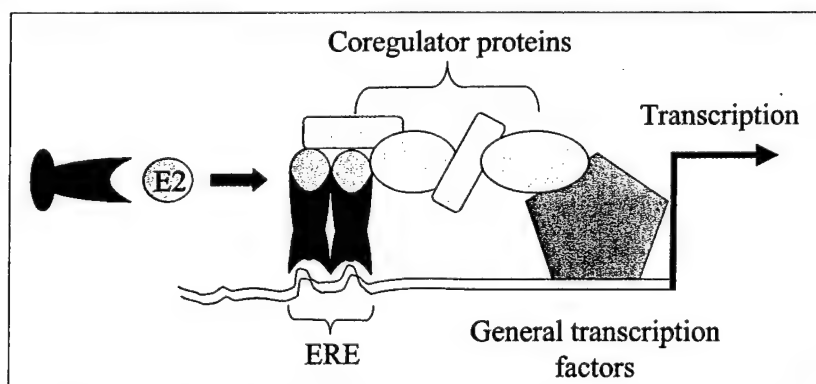

Figure 1: How the oestrogen receptor works. The receptor binds ligand, then binds DNA as a dimer. Other coregulator proteins are recruited, such that the oestrogen receptor and its associated proteins ultimately regulate the activities of the cell's general transcription apparatus assembled at the gene's promoter.
Ligand = 17β-estradiol, any phyto-oestrogen, or xeno-oestrogen; ER=oestrogen receptor; ERE=oestrogen responsive element. GTA = general transcription apparatus.

Population	Exposure	Association[1]	Citation
212 patients (cases) 212 hospital (controls)	total soybean products	none	Hirohato *et al.* (*J Natl Cancer Inst Monogr 69*:187–190, 1985)
534 patients (cases) 534 community (controls)	total soy protein	none	Yuan *et al.* (*Br J Cancer 71*:1353–1358, 1995)*
300 patients (cases) 300 community (controls)	total soy protein	none	Yuan *et al.* (*Br J Cancer 71*:1353–1358, 1995)
86 spouses of patients	weekly intakes	tofu[2]: none miso soup: p=0.046	Nomura *et al.* (*Am J Clin Nutr 31*: 2020–2025, 1978)
1,186 patients (cases) 23,163 (controls)	miso soup	pre[3]: OR1.16 (0.98; 1.37) (borderline increased risk)	Hirose *et al.* (*Jpn J Cancer Res 86*: 146–154, 1995)
222 patients (cases) 222 sisters (controls)	tofu intake	OR=0.5 (0.2-1.1) (borderline protective effect)	Witte *et al.* (*Breast Cancer Res Treat 42*:243–251, 1997)
200 patients (cases) 420 hospital (controls)	soy protein	post: none pre: -ve	Lee *et al.* (*Lancet 337*: 1197–1200, 1991)
597 patients (cases) 996 community (controls)	tofu intake	post: none pre: OR=0.84 (0.70, 0.99) all cases: OR=0.83 (0.72, 0.95)	Wu *et al.* (*Cancer Epidemiol Biomarker Prev 5*: 901-906, 1996)
Meta analysis	N/A	pre: OR=0.80 (0.71–0.90)	White, Hilakivi-Clarke, Clarke, Trock: submitted

Table 2. Soy epidemiological studies. While neither a complete list, nor a full description of the data in each study (which would be beyond the scope of this article) these data demonstrate the variability in the epidemiological literature regarding the association between soy consumption and breast cancer risk. The meta analysis, which derives an overall estimate by combining all studies, includes some reports not in this table.
[1] Most studies performed logistic regression and obtained an odds ratio (OR) to describe the association between exposure and breast cancer risk. The OR is an approximation of the relative risk and is presented with its 95% confidence interval. In these studies, the association is protective when the OR <1 and indicates an increase in breast cancer risk when the OR >1. Generally, the OR is significant when the confidence interval does not encompass 1.
[2] Generally, tofu has a higher isoflavone content than soy drinks, but levels comparable to, or slightly lower than, miso and other Japanese soups.
[3] Pre=premenopause; post=postmenopause.

breast cancer risk? In eastern countries, soy exposure is likely to be throughout life. This should have either no effect, or even be modestly protective. Feeding soy to infant girls might reduce their breast cancer risk in later life. We may not have to wait a lifetime to find out. Soy-based infant formulas (baby milk) have been widely available and extensively used for several decades. These contain very high levels of phyto-oestrogens (Setchell *et al.*, 1997). The children exposed to these formulas are already becoming old enough to determine whether there is a reduction in premenopausal breast cancer, if the data can be obtained.

Zearalenone
The mycotoxin zearalenone also fulfils our definition of an oestrogen. Zearalenone has been used as a contraceptive, as an oestrogen replacement therapy for postmenopausal women, and as an anabolic agent to enhance growth in cattle and lambs. The growth of MCF-7 human breast cancer cells *in vitro* is stimulated by zearalenone. This mycotoxin has also been reported to enlarge the mammary gland and induce spontaneous mammary tumours in mice. In rat studies, we found that *in utero* exposure to zearalenone did not affect susceptibility of the female pups to mammary carcinogenesis. However, like genistein, prepubertal exposure was protective.

What is so different between zearalenone and genistein?

Why these differences between genistein and zearalenone occur, and why timing is so important, is not entirely clear. One possibility is that coregulators recruited into the ER protein complex (Figure 1) could differ depending on the ligand. We also should not exclude the possibility that the oestrogenic effects of these compounds reflect differences in how they activate ERα and ERβ. For example, genistein has a much higher affinity for ERα than zearalenone (Table 3), while oestradiol has a similar affinity for both ERα and ERβ. These data suggest that not all oestrogens are created equal.

Environmental oestrogens (xeno-oestrogens)
Perhaps the most widely studied xeno-oestrogens are the organochlorines. These compounds are ubiquitous in the environment. The organochlorines are lipophilic (fat loving), slow to metabolise, and persist in adipose tissues. They can accumulate up the food chain and into the human diet. Their widespread occurrence and tendency to bioaccumulate, has raised concern that they may produce chronic, low-level oestrogenic stimulation, resulting in an increased risk of breast cancer.

The most widely implicated organochlorines are the chlorinated pesticides, *e.g.*, DDT (Figure 2) and its metabolites, the polychlorinated biphenyls (PCB), and the polychlorinated dibenzo compounds (PCD). DDT was first produced as an insecticide more than 50 years ago. While banned in the US in 1972, DDT is still commonly used in many developing countries. It is readily metabolised to the more stable and lipophilic DDE. Other implicated chlorinated pesticides include kepone, methoxychlor, hexachlorobenzene, hexachlorocyclohexane, chlordane, toxaphene, aldrin and dieldrin.

The PCBs have been produced commercially for more than 60 years for uses such as flame retardants, insecticides and lubricants. Their production was discontinued in the US in 1977. Contamination of the environment continued to occur through waste disposal and leakage. While not commercially manufactured, PCDs occur as contaminants and by-products in a number of production and combustion processes.

DDT, DDE and many PCBs have been found in breast milk, serum and adipose (fat) tissue. The ability to detect these compounds in human milk suggests that they may accumulate in breast adipose tissue and could be passed to breast-fed infants. In contrast, PCDs are found at very low levels in human tissue or blood. Discontinuation of the use of the chlorinated pesticides and PCBs is being reflected in decreasing levels in most of the US population.

Other possible xeno-oestrogens have recently begun to attract attention. Among the most potent are bisphenol A (which is used in the manufacture of polycarbonate) and octylphenol (Figure 2). Bisphenol A can leach into foods from packaging.

Isoflavones	Lignans	Lactones	Stilbenes	Coumestans
soy beans, beer, bourbon, peas, fruit	grains, seeds, berries, nuts	Barley, wheat, corn, rice, peas, seeds	grapes wines	soy beans peas, beans

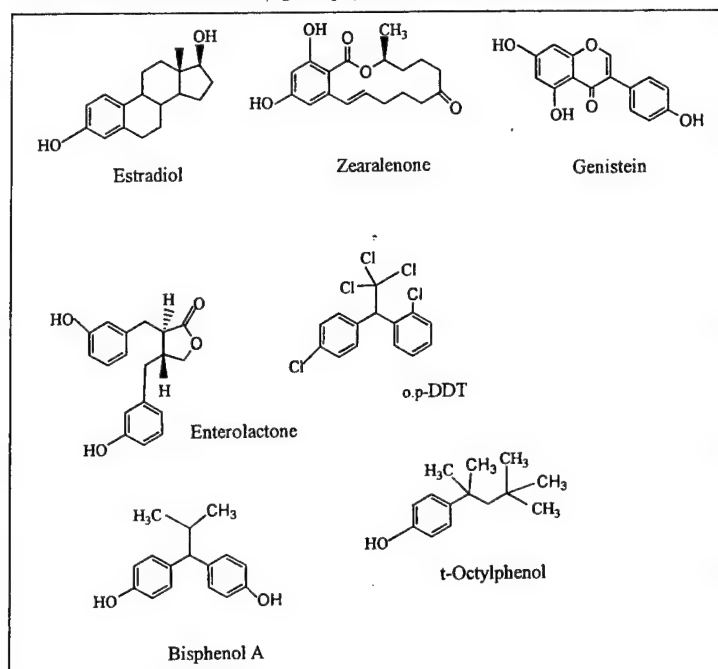**Table 1.** Some sources of oestrogenic phytochemicals (phyto-oestrogens).

Figure 2: Structure of 17B-estradiol and selected phyto-oestrogens and xeno-oestrogens. The compounds in this figure and / or in the text are:

bisphenol A = 4,4'isopropylidenediphenol
daidzein = 4',7-dihydroxyisoflavone
DDE = 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene
DDT = 1,1,1-trichloro-2-2-bis(4-chlorophenyl)ethane
17B-estradiol = 1,3,5-estratriene-3,17B-diol
genistein = 4',5,7-trihydroxyisoflavone
octylphenol = 4-tert-octylphenol
resveratrol = trans-3,4',5-trihydroxystilbene
zearalenone = 2,4-dihydroxy-6-[10-hydroxy-6-oxo-trans-1-undecenyl]-B-resorcyclic acid-μ lactone

who excrete high levels of lignans have a lower breast cancer risk. Excretion levels tend to be high in some vegetarians and populations that consume high amounts of whole grain products (Adlercreutz, 1990).

Studies of soy and breast cancer risk

Several case-control studies have explored the soy/ breast cancer hypothesis but the data are often contradictory or unclear. Four out of eight studies found no statistically significant association. One small study found an association for Japanese soup but not for tofu, despite both foods having comparable genistein/ isoflavone levels. The lack of an association with tofu consumption was subsequently contradicted, with a significant effect in both premenopausal and postmenopausal women being reported. However, another study failed to find any association with soy protein intake and breast cancer risk in postmenopausal patients, reporting a protective association only in premenopausal women (see Table 2).

We have recently combined these studies using a statistical technique called meta analysis. This allowed us to explore the data to determine if there were any clear associations not apparent from a simple reading of the individ-

ual studies. The results from this analysis found no effect on postmenopausal breast cancer risk, and only a small (20%) reduction in the risk of developing breast cancer in the premenopause (Table 2). In contrast, the beneficial effects of soy consumption on reducing the risk of cardiovascular disease are much clearer (Potter, 1995).

Lab trials indicate a mixed influence. Some animal studies find a protective effect, some no effect, and some a significant increase in breast cancer risk associated with soy/ genistein consumption. Genistein also stimulates human breast cancer cell proliferation *in vitro* and can support the growth of human breast cancer xenografts in immunodeficient mice (Hsieh *et al.*, 1998). Human volunteers fed soy milk experienced changes in their menstrual cycling consistent with a potent oestrogenic exposure. Soy consumption also induces clearly oestrogenic changes in women's breast nipple aspirate fluid. These symptoms might suggest an increase in breast cancer risk.

Genistein and the timing of exposure

Why should genistein produce such diverse and potentially conflicting results, and what might the potential risks or advantages be of exposure? We and others have begun to look at the timing of exposure, to determine whether this can affect breast cancer risk.

Oestrogens are required for the successful maintenance of pregnancy, the levels increasing throughout pregnancy to peak at birth. The concentrations of oestrogens vary considerably among pregnant women. The cause of this variability is not known but may be dietary. There is some evidence that higher oestrogen levels during pregnancy are associated with daughters' increased breast cancer risk. Daughters of mothers who suffered from pre-eclampsia/ eclampsia during pregnancy, which is associated with low levels of oestrogens, have a lower breast cancer risk. Conditions associated with high oestrogen levels (such as high birth weight and infant jaundice) lead to higher risks.

Administering oestradiol (an ovarian oestrogen) or genistein to pregnant rats increases the susceptibility of their female offspring to chemically-induced mammary cancers. In addition, the age of onset of sexual maturation is accelerated (Hilakivi-Clarke *et al.*, 1997). These exposures increase the number of structures within the mammary glands known to be targets for chemical carcinogens. The more targets, the greater the probability that one will be transformed and develop into a breast tumour.

The situation is quite different if the exposure to genistein occurs after birth but before sexual maturation. When genistein is administered during this period, female rats have a reduced susceptibility (Murrill *et al.*, 1996; Hilakivi-Clarke *et al.*, 1999). Data indicate that this exposure is sufficient to override the increased risk associated with exposure during pregnancy. When considered together it is clear that timing is everything.

How do these studies relate to human exposures and

Do xeno-oestrogens affect breast cancer risk?

The long-term, gradual increase in breast cancer incidence in the US and EU has been put forth as evidence for risk from xeno-oestrogen exposure. However, this increase has occurred during a time of changes in reproductive patterns, diet, and occupational roles for women. Each of these could also influence breast cancer risk. The low level of breast cancer among Japanese women, despite a high body burden of organochlorines, has been cited as evidence against the xeno-oestrogen hypothesis. However, the positive effects of Japanese diet and reproductive patterns may overcome a small risk from organochlorines, if such a risk existed. Overall, there is no convincing evidence that the oestrogenic effects of organochlorines increase the risk of human breast cancer.

Case-control studies have drawn the most attention and controversy. These are studies in which biological specimens from women with breast cancer (cases) are compared with those from healthy women (controls). There have been twelve case-control studies to date but only five had reasonably adequate sample sizes.

Of the four methodologically strongest studies, two observed a statistically significant result, while two did not. At this time, there is no clear indication that exposure to organochlorine pesticides or PCBs is a significant risk factor for breast cancer. Citations for these trials can be found in (Clarke *et al.*, 1998).

Phyto-oestrogens		
Compound	ERα RBA[1]	ERβ RBA
Zearalenone	1.2×10^{-2}	ND
Zearalenone (zearalenone metabolite)	0.2	0.2
Genistein	0.05	0.36
Coumestrol	0.94	1.84
Resveratrol	10^{-4}	ND
Xeno-oestrogens		
p,p'-DDE	10^{-5}	ND
Bisphenol A	5×10^{-4}	3.3×10^{-3}
Methoxychlor	10^{-4}	1.3×10^{-4}
Octylphenol	10^{-3}	ND
β-hexachlorohexane	4×10^{-4}	ND
Butylated hydroxyanisole	10^{-6}	ND
Estimated Relative Oestrogenic Exposures[2]		
Contraceptive pill	100%	
Hormone replacement therapy	20%	
Phyto-oestrogens	6%[3]	
Xeno-oestrogens	1.5×10^{-8}%	

Table 3. Relative oestrogenic potencies and exposures. The data are derived from several sources. ND = no data.
[1] RBA = relative binding affinity based on the Kds as estimated by Martin *et al.* (*Endocrinology* **103**: 1860-1867, 1978; Kuiper *et al.* (*Endocrinology* **138**: 863-870, 1997) and Gehn *et al.* (*Proc Natl Acad Sci USA* **94**: 14138-14143, 1997) where 17β-estradiol = 1.
[2] These are primarily derived from the mass balance estimates of Safe (*Environ Health Perspect* **103**: 346-3512, 1995) taking the oestrogenicity of the contraceptive pill = 100%.
[3] The estimate for phyto-oestrogens is based on an average RBA = 10^{-3}. This could be higher if the population is primarily exposed through soy or zearalenone/zearalenol and possibly lower in other populations.

Oestrogens versus phyto- versus xeno-oestrogens

In premenopausal women, the ovarian oestrogens are likely to provide the major source of oestrogenicity. Phyto-oestrogens, particularly where the exposure is substantial, could provide sufficient oestrogenicity to affect ovarian function. Such an effect might reduce both natural oestrogen levels and breast cancer risk.

In postmenopausal women, in western populations, oestrogen biosynthesis in adipose tissues, and any hormone replacement therapy, probably provide the main oestrogenic exposure. However, exposure to some phyto-oestrogens may be sufficient to provide an almost equivalent contribution to oestrogenicity. In postmenopausal women in Asia, when hormone replacement therapy is not administered, the oestrogenicity of phyto-oestrogens may predominate.

In some pre-existing breast tumours, the levels of the natural oestrogens are relatively high because the tumours can synthesise oestrogens from adrenal androgen precursors. The potentially protective effects associated with soy consumption suggest that genistein, or another component of soy, might function as an antiproliferative or even an anti-oestrogen. However, there is little experimental evidence for genistein functioning as anything other than a mitosis-inducing oestrogen in this regard. Assuming that soy and/ or genistein contribute to low Asian incidence, it may be the lifetime exposure and its effects on mammary gland development that are most important.

The low affinities and exposure for xeno-oestrogens, and the lack of any clear and compelling epidemiological evidence, do not support a major affect on breast cancer risk. Despite their potentially greater availability and persistence, the concentrations of free compound likely to be accessible for ER binding are probably not sufficient to compete effectively with the concentrations of natural- and phyto-oestrogens. Thus, any effect of these compounds is unlikely to be related to their oestrogenicity.

Conclusions

There is a difference between risk and 'cause' in cancer. Oestrogens could be chemical carcinogens. Some natural oestrogen metabolites are highly chemically reactive and can damage DNA. However, it is not clear whether this damage alone is sufficient to cause cancer (Clarke *et al.*, 1992). It is most likely that the effects we have discussed reflect either promotional activities, *i.e.*, promoting the survival and proliferation of cancerous cells, or pre-initiation effects, *i.e.*, changes in the susceptibility of normal tissues to transformation.

We have suggested that the xeno-oestrogens contribute little to affecting breast cancer risk. We believe this to be a reasonable conclusion, given what we currently know about the pharmacology of these compounds. The phyto-oestrogens may contribute to breast cancer risk. However, whether the influence is protective or destructive will likely depend on the nature, timing and dose of exposure.

Oestrogenicity is not only associated with breast cancer risk: there is good evidence to suggest that oestrogen or soy intake may reduce the risk of cardiovascular disease; apparent reduction in sperm counts has been attributed to increased xeno-oestrogen exposure; and changes in the patterns of sexual differentiation of various reptiles may reflect contamination of the environment with oestrogenic compounds. Not surprisingly, there has been considerable

interest in hormonally active compounds. It seems likely that additional phyto- and xeno-oestrogens will be identified in the next few years. Understanding the precise importance of exposure to these agents may take a little longer.

Acknowledgements

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http://www.som.tulane.edu/ecme/eehome/
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http://www.epa.gov/scipoly/oscpendo/index.htm
Environmental Protection Agency – the endocrine disruptor screening program web site. This web site provides information about the endocrine system and why certain chemicals can affect it, how the EPA Endocrine Disruptor Screening Program was developed, and the current status of EPA's implementation activities.

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Alcohol exposure in utero and breast cancer risk later in life.

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Hypothesis:
Alcohol exposure in utero and breast cancer risk later in life

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Since the pioneering work of Hiatt and Bawol (1984), there has amassed a considerable amount of evidence that moderate to heavy alcohol consumption increases risk of breast cancer in women (Longnecker, 1999; Willett et al. 1987). A plausable mechanism is by alcohol's effects on circulating hormone levels. Alcohol administration has been reported to increase circulating estradiol levels in premenopausal women (Reichman et al., 1993); the evidence is mixed in postmenopausal women (Purohit, 1998). In a cross-sectional study of premenopausal women, Muti et al. (1998) determined an association between reported alcohol consumption and serum estradiol levels during the luteal phase of the menstrual cycle, and found an 18% elevation in drinkers, consuming an average 1 drink per day, compared to abstainers. It is not clear how alcohol affects circulating estradiol levels. Alcohol has been reported to increase aromatase activity; i.e., the conversion of testosterone to estrogens, resulting in reduced testosterone and increased estrogen levels (Gavaler & Van Thiel 1992). Alcohol also might interact with luteinizing hormone production from the pituitary (Rettori & McCann 1987), resulting in increased estradiol release from the ovaries.

In addition to affecting estrogen levels, alcohol appears to influence melatonin. Alcohol administration has been reported to reduce the nocturnal rise in serum melatonin in rats (Moss et al. 1986), and in humans (Ekman et al., 1993; Rojmark et al., 1993) under experimental conditions. In a large cross-sectional study, Stevens et al. (in press) found a significant inverse association of alcohol consumption and urinary 6-sulphatoxymelatonin, a good indicator of nocturnal blood levels (Cook et al., 2000), in healthy women living under normal conditions in the Seattle area. Importantly, there was no effect of one drink on melatonin level, but a 9% reduction with 2 drinks, 15% with 3 drinks, and 17% with 4 drinks or more.

It may be that an increase in circulating estradiol levels and a reduction in melatonin levels after alcohol exposure, are not just simultaneous events, but causally related. Stevens and Hiatt (1987) suggested that alcohol ingestion may result in lowered melatonin levels which, in turn, may lead to elevated circulating estradiol concentration in blood (Cohen et al., 1978). Specifically, decreased concentrations of melatonin might increase release of gonadotrophins, leading to an increase in ovarian estrogen production (Voordouw et al. 1992; Penny et al., 1987; Brzezinski, 1997; Kauppila et al, 1987).

Both high estrogen levels and low melatonin levels have been implicated in increasing the risk to develop breast cancer. Findings in human breast cancer cells growing in culture, in animal models (Clarke et al. 1992), and in epidemiological studies, at least in postmenopausal women (Key 1999; Kabuto et al., 2000), link elevated circulating estrogen levels to increased breast cancer risk. In addition, in vitro and in vivo data in animals indicate that melatonin suppresses malignant growth (Cos & Sanchez-Barcelo 2000). Findings in humans also support the notion that melatonin reduces breast cancer risk (Feychting et al. 1998; Verkasalo et al. 1999).

These observations can be considered in the context of the hypothesis that an elevated exposure to estrogens in utero will increase lifetime risk of breast cancer (Trichopoulos, 1990) by altering normal breast development. This hypothesis was subsequently supported in a number of epidemiological studies (Potischman and Troisi 1999). Animal models also support the hypothesis and show that elevated in utero estradiol levels lead to altered mammary gland development (Hilakivi-Clarke et al., 1997). Further, an exposure of pregnant rats to estradiol, or feeding them a diet high in n-6 polyunsaturated fatty acid (PUFA), which significantly raises circulating estradiol levels, results in increased DMBA-induced mammary tumor incidence in their female offspring.

For women who are pregnant, ingestion of alcohol may lead to elevated circulating estradiol levels, either through a reduction of melatonin or some other mechanism. This may then affect the developing mammary tissue such that the lifetime risk of breast cancer is raised in their daughters.

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TALLNESS AND OVERWEIGHT DURING CHILDHOOD HAVE OPPOSING

EFFECTS ON BREAST CANCER RISK

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Summary

Body size in adult life is associated with breast cancer risk. Pre-menopausal women who have low body mass are at increased risk of developing the disease. After the menopause, greater body weight and tallness increase the risk. We studied how weight and height during childhood affect breast cancer risk. Birth and school health records containing information on maternal, neonatal, and childhood measurements of body size were obtained for 3,447 women born during 1924-33 at the University Hospital of Helsinki, Finland. Through linkages with the National Hospital Discharge Registry and the Cause of Death Registry women who developed breast cancer during 1971 - 1995 were identified. We found that 177 women had been admitted to hospital with breast cancer, of whom 49 had died from the disease. 135 (76%) of these women were aged 50 years or more at the time of diagnosis, and were therefore likely to have been post-menopausal. We examined the trends in hazard ratios for breast cancer with measurements of body size at birth and during childhood. Hazard ratios for breast cancer rose with increasing weight and length at birth, though neither trend was statistically significant. At each age, from 7 to 15 years, the girls who later developed breast cancer were on average taller and had lower body mass than the other girls. Unadjusted hazard ratios rose across the range of height (p = 0.01 at age 7 years) and fell across the range of body mass index (p = 0.009 at age 7 years). In a simultaneous analysis the hazard ratio for breast cancer was 1.27 (95% CI 0.97 – 1.78, p = 0.08) for every kilogram increase in birth weight and 1.21 (95% CI 1.06 – 1.38, p = 0.004) for every kg/m^2 decrease in body mass index at 7. Our findings indicate that tallness in childhood is associated with increased risk of developing breast cancer. One possible explanation is that tall childhood stature reflects high plasma concentrations of insulin-like growth factors which, persisting throughout life, may result in an increased vulnerability to breast cancer. In contrast, we found that being overweight in childhood reduces breast cancer risk. The increased adipose tissue -derived estrogen levels in overweight children could induce early breast differentiation and eliminate some undifferentiated mammary epithelial cells as targets for malignant transformation.

Key words: Breast cancer - body mass - height - childhood

Introduction

It has been hypothesised that accelerated growth during fetal life or an enhanced estrogenic environment *in utero* increases subsequent breast cancer risk (Trichopoulos 1990). This hypothesis is supported by limited indirect epidemiological evidence, including studies showing that high birth weight and being a dizygotic twin, both of which are associated with elevated pregnancy estrogen levels (Gerhard et al 1987; Johnson et al. 1994), increase the risk of developing breast cancer (Braun et al 1995; Cerhan et al 2000; Michels et al 1996; Sanderson et al 1996; 4-7). Animal studies show that maternal exposure to estradiol during pregnancy or a high fat diet that elevates pregnancy estradiol levels, increase carcinogen-induced mammary tumorigenesis among female offspring (Hilakivi-Clarke et al 1997).

Relatively little is known about growth during childhood and breast cancer. Tall or obese women are at an increased risk of developing postmenopausal breast cancer (Cold et al 1996; Vatten and Kvinnsland1990; Yong et al 1989; Ziegler 1997). Thin women are more likely than obese women to develop pre-menopausal breast cancer (Cleary and Maihle 1997; Huang et al 1997; Potischman et al 1996; Trentham-Dietz et al 1997). Three recent studies suggest that high childhood or adolescent body mass index may protect against breast cancer risk (Berkey et al. 1999; Le Marchand et al 1988; Magnusson et al 1998). Many investigators, however, believe the opposite and propose that childhood obesity increases the risk (Colditz and Frazier1995; deWaard and Trichopoulos 1988; Hunter and Willett 1996). This belief is based on the association between childhood obesity and early onset of puberty (Frisch and McArthur 1974), since early puberty is consistently related to increased risk of breast cancer (Hulka and Stark 1995).

We have studied a cohort of 3,447 Finnish women whose size at birth and childhood growth were recorded, to examine whether height and body mass index during childhood affected breast cancer risk. We also examined whether childhood growth modified the effects of birth size on risk.

Methods

The study cohort comprised women who were born at the Helsinki University Central Hospital during 1924 – 1933, who went to school in the city of Helsinki, and were still residents in Finland as of 1971. The eleven year birth period chosen gives a cohort of women old enough to have experienced pre- and post-menopausal breast cancer. Details of the birth records kept by the Helsinki University Central Hospital have been described previously (Forsen 1997). School health records for all children in Helsinki are stored in the city archive. Beginning in 1971, all residents of Finland have been assigned a unique personal identification number. From the birth and school health records and identification numbers, we identified 3,688 women for use in our study (Forsen 1999). Of these women, 241 subsequently emigrated and the date of emigration was not always recorded. We therefore excluded them from the study to leave 3,447 women.

Recorded data on the new-born babies included birthweight, length, head circumference and placental weight. Data on their mothers included age, parity, height and date of the last menstrual period, together with body weight, measured on admission in labour. The school records included an average of 10 (± SD 4) measurements of height and weight between the ages of 6 and 16 years, recorded during periodic medical examinations. Age at menarche was not recorded. Further, no information was available of the factors after menarche that are known to be related to breast cancer risk, i.e., age at menopause, adult body weight, parity, or age at first pregnancy.

By using the personal identification number, we identified all hospital admissions and deaths among the women during 1971 – 1995. All hospital admissions in Finland are recorded in the national hospital discharge register. All deaths are recorded in the national mortality register. Causes of hospital admissions or death were recorded according to ICD-8 (international classification of diseases, 8th revision) until 1986; thereafter ICD-9 was used until 1995. The first three digits from the cause of admission or death were used to identify breast cancer cases in the cohort (174 in ICD-8 and ICD-9).

Statistical methods   We examined the trends in hazard ratios with maternal, neonatal and childhood measurements. Tests for trend were based on Cox's proportional hazards model. Each measurement of height, weight and body mass index for each girl was converted to a Z score *(Royston 1991)*. We then interpolated between successive Z scores with a piecewise linear function and so obtained a Z score at each birthday from age 7 to 15 years. The Z scores were back transformed to obtain the corresponding height, weight and body mass index at these ages, as previously described *(Forsen 1999)*. Growth velocity was measured as the change in Z-scores between ages 7 and 15 years.

Results

Maternal, neonatal and childhood characteristics of the 3,447 women are shown in Table 1. We found that 177 of these women were admitted to hospital with breast cancer, of whom 49 died from the disease. The annual death rate from breast cancer at ages 45 to 64 years was 6.1 per 10,000. In 1971, when the first breast cancer cases were ascertained, the women's ages ranged from 38 to 47 years. Although most women would have been pre-menopausal at that time, 135 (76%) of the 177 women who developed breast cancer during the follow-up, were aged 50 years or more at the time of diagnosis and were therefore likely to have been postmenopausal. In the analyses which follow we found no differences in the association with breast cancer first diagnosed below and over the age of 50 years.

Size at birth   Table 2 shows hazard ratios for breast cancer according to size at birth. The ratios tended to rise with increasing birthweight, although this was not statistically significant. The trend with birth length was similar, though also not significant. Hazard ratios were not related to placental weight nor to the length of gestation. The trends in Table 2 were little changed by adjusting for gestation.

Growth in childhood   Figure 1, based on the Z scores, shows that at each age from 7 to 15 years the women who developed breast cancer were, on average, taller than the other women (p<0.05 at every age). At 7 years, for example, their height was 0.8 cms above the average, while at 15 years it was 1.3 cms above. In contrast, women who developed breast cancer were thinner than the other women at all ages from 7 to 15 years (p<0.05 at each age). Their body mass index at 7 years was 0.3 kg/m^2 below the average and at 15 years it was 0.4 kg/m^2 below the average. The changes in Z-score for height, weight and body mass index from 7 to 15 years were not statistically significantly different from those of the other women.

Table 3 shows hazard ratios for breast cancer according to height and body mass index at 7 and 15 years. The trends for increased breast cancer risk with increasing height and falling body mass index were both statistically significant. Body mass index at 7 years was not strongly correlated with birthweight (correlation coefficient = 0.15). In a simultaneous analysis, the hazard ratio for breast cancer was 1.21 (95% CI 1.06 – 1.38, p = 0.004) for every kg/m^2 decrease in body mass index at 7 years and 1.27 (95% CI 0.97 – 1.78, p = 0.08) for every kilogram

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increase in birthweight. There was no interaction between birthweight and body mass index in their effect on breast cancer risk.

Maternal characteristics   As expected, taller mothers tended to have taller daughters, the correlation coefficient between mothers and daughter's height at seven being 0.36; and mothers with higher body mass indices had daughters with higher body mass indices, correlation coefficient 0.23 at age 7 years. However, mothers' heights, weights and body mass indices during pregnancy were unrelated to the occurrence of breast cancer in their daughters. Similarly, the mothers' ages and parities were not related to their daughters' breast cancer risk.

Discussion
The present study investigated associations between early growth and breast cancer risk in a cohort of 3,447 Finnish women, born in Helsinki, most of whom developed the disease after the age of 50 years, that is post-menopausally. We found that high birth weight and long body length at birth were associated with increased risk of the disease, although the association did not reach statistical significance. These borderline associations are similar to those found in a cohort of Swedish women born in Uppsala (Ekbom et al 1992).

In addition to size at birth, we were able to examine how growth between 7 and 15 years modified the risk of breast cancer. We found that women who developed the disease had above average height through childhood but were relatively thin, with a below average body mass index. We did not have information on adult body mass index, and it is possible that these associations with childhood height and body mass are mediated through effects of adult body mass on breast cancer risk. However, it is clear that childhood body mass did not mediate the effects of high birthweight, since they had independent and opposite effects on breast cancer risk.

Earlier studies have shown that, after the menopause, tall women are more prone to develop breast cancer than shorter women (Cold et al 1996; Vatten and Kvinnsland1990; Ziegler 1997). Our data show that the association of tallness with breast cancer may reflect a greater height from birth onwards. Increased breast cancer risk was associated with increased length at birth followed by above average height at every age from 7 to 15 years. Previously, the Nurses' Health Study (Berkey et al 1999) found an association between high peak height growth velocity, the most growth attained during any single year of adolescence, and an increased risk of breast cancer. The observation, however, was based on estimates of growth velocity derived from recalled body fatness at 10 years, menarcheal age and adult height. The results of our study, which are based on actual measurements of height during childhood, show that the velocity of height growth does not predict breast cancer risk.

Girls who are tall during childhood tend to have an earlier menarche than short girls (Ellison 1981; Marshall and De Limongi 1976) and our observations that tall girls exhibit increased breast cancer risk may partly explain the association between breast cancer and early menarche (Hulka and Stark 1995). Greater height in childhood is associated with higher plasma concentrations of insulin-like growth factor -1 (IGF-1) (Juul and Skakkebaek 1997; Nilsson et al 1994), and this growth factor plays an important role in determining onset of puberty (Hiney et al 1996; Juul et al 1995). Furthermore, high plasma IGF-1 concentrations in pre-menopausal women are associated with increased breast cancer risk (Hankinson et al 1998). IGF-1 is therefore a possible link between childhood height, age at menarche and breast cancer.

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Puberty occurs when a girl reaches a critical weight/body mass, and overweight girls tend to experience menarche earlier than girls with normal body weight (Frisch and McArthur 1974). It might be expected that overweight girls would have increased breast cancer risk. However, neither earlier prospective data (Le Marchand et al 1988), nor retrospective data (Berkey et al 1999; Magnusson et al 1998) support this. Two retrospective case-control studies were based on information about childhood body mass as it was recalled during adult life before or after breast cancer diagnosis (Berkey et al 1999; Magnusson et al 1998). It is important to note that few girls in Finland were obese sixty years ago, when women in our cohort were children. The highest BMI category at age 15 was > 21.5 kg/m^2, which according to present standards represents normal body size. We cannot therefore conclude that childhood obesity, as defined in clinical practice today, protects against breast cancer.

Recent animal studies may provide clues to the biological mechanisms that link high childhood body mass index to a low risk of breast cancer. In animals exposed to estrogens in early life, the mammary glands may differentiate early and are less susceptible to developing tumours upon exposure to carcinogens (Grubbs et al 1985; Nagasawa et al 1974). In humans body mass index is an indicator of estrogenicity, especially before puberty when adipose tissue is the major site of estrogen release. Perhaps being overweight in childhood induces early breast differentiation and eliminates some undifferentiated mammary epithelial cells as targets for malignant transformation. Another possibility is that the estrogenic effects of high childhood body mass increase the expression of tumour suppressor genes, such as BRCA1 (Hilakivi-Clarke 2000). This gene is known to be overexpressed during puberty (Marquis et al 1995). It is induced by estrogens (Gudas et al 1995; Spillman and Bowcock 1996) and its activity is associated with increased breast differentiation (Rajan 1996), maintenance of the genomic integrity and DNA repair (Gowen 1998).

In conclusion, we have found that women who developed breast cancer were tall and thin at all ages from 7 to 15 years. We speculate that these associations reflect the effects of high plasma concentrations of insulin-like growth factors, and a protective effect of high body mass through early breast differentiation.

Contributors: All the authors took part in the design and analysis of the study and jointly wrote the paper.

Competing interests: None declared.

Table 1: Maternal, neonatal and childhood characteristics of 3,447 women born at Helsinki University Central Hospital during 1924-33.

	Mean (SD)	Range	No of missing values
Maternal			
Height (m)	1.58 (0.06)	1.04 - 1.86	245
Weight in late pregnancy (kg)	66.8 (8.7)	45 – 134	261
Body mass index in late pregnancy (kg/m^2)	26.7 (3.1)	19.4 – 51.1	285
Age (years)	27.7 (5.8)	15 – 48	9
Primiparous (%)	42.0		3
Neonate			
Birthweight (g)	3315 (493)	1470 – 5600	0
Head circumference (cm)	34.3 (1.4)	28.0 – 39.0	10
Birth length (cm)	49.7 (2.0)	38.0 – 59.0	12
Ponderal index (kg/m^3)	26.9 (2.4)	15.6 – 50.0	12
Placental weight (g)	628 (125)	200 – 1290	7
Length of gestation (days)	276 (15)	197 - 307	179
% born before 37 weeks gestation	10.1		179
Child			
Height (m) at age 7 years	1.19 (0.05)	0.98 - 1.37	0
At age 11 years	1.39 (0.06)	1.13 - 1.61	0
At age 15 years	1.58 (0.06)	1.29 - 1.81	0
Weight (kg) at age 7 years	21.7 (2.9)	14.1 - 37.9	0
at age 11 years	32.1 (5.1)	18.2 – 64.5	0
at age 15 years	49.7 (7.7)	26.2 – 92.9	0
Body mass index (kg/m^2) at age 7 years	15.3 (1.3)	11.7 – 24.1	0
at age 11 years	16.5 (1.7)	12.2 – 31.2	0
at age 15 years	19.8 (2.5)	12.3 – 41.2	0
No. of people in house	4.5 (1.5)	2 - 14	570
No. of rooms in house	1.7 (0.8)	1 - 7	536
Crowding (no. of people/no. of rooms)*	2.8 (1.6)	0.6 - 11	576

* Log transformed

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Table 2:   **Hazard ratios for breast cancer according to size at birth**

Variable	No of cases	No. of women	Hazard ratio (95% CI)
Birthweight (g)			
≤2500	6	191	1.0
- 3000	31	708	1.4 (0.6 – 3.4)
- 3500	84	1420	1.9 (0.8 – 4.3)
- 4000	41	880	1.5 (0.6 – 3.5)
>4000	15	248	1.9 (0.7 – 5.0)

Hazard ratio per kg = 1.22 (0.90 to 1.65) p = 0.2

Variable	No of cases	No. of women	Hazard ratio (95% CI)
Birth length (cms)			
< 48	27	726	1.0
- 49	30	595	1.4 (0.8 – 2.3)
- 50	65	1077	1.6 (1.0 – 2.5)
- 51	32	588	1.5 (0.9 – 2.5)
>51	23	449	1.4 (0.8 – 2.4)

Hazard ratio per cm = 1.06 (0.98 to 1.15) p = 0.13

Table 3:   **Hazard ratios for breast cancer according to body size at 7 and 15 years**

| Variable	No. of cases	No. of women	Hazard ratio (95% CI)
Height at age 7 (cms)			
≤114.5	22	641	1.0
- 117.5	32	703	1.3 (0.8-2.3)
- 120	39	694	1.7 (1.0-2.8)
- 123	41	722	1.7 (1.0-2.9)
>123	43	687	1.9 (1.1-3.1)
p for trend = 0.01			
Height at age 15 (cms)			
≤153	23	657	1.0
-157	34	750	1.3 (0.8-2.2)
-160	33	720	1.3 (0.8-2.2)
-163	38	604	1.8(1.1-3.1)
>163	49	716	1.9(1.2-3.2)
p for trend = 0.005			
BMI at age 7 (kg/m^2)			
≤14.3	46	701	1.9 (1.2-3.1)
- 14.9	34	682	1.4 (0.8-2.4)
- 15.5	45	715	1.8 (1.1-2.9)
- 16.2	27	642	1.2 (0.7-2.1)
>16.2	25	707	1.0
p for trend = 0.009			
BMI at age 15 (kg/m^2)			
≤18	49	769	1.7(1.0-2.7)
-19	33	596	1.4(0.9-2.4)
-20	38	649	1.5(0.9-2.4)
-21.5	29	724	1.0(0.6-1.7)
>21.5	28	709	1.0
p for trend = 0.03			
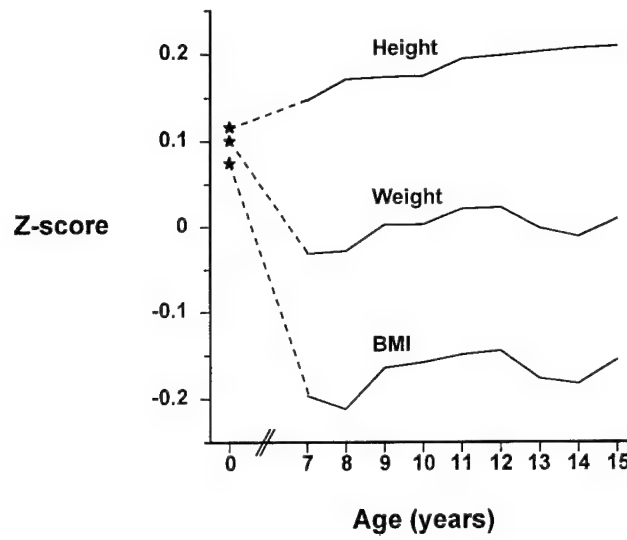
Figure legend

Figure 1: Height, weight and body mass index during childhood, expressed as standard deviation (Z) scores, in women who later developed breast cancer

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DO ESTROGENS ALWAYS INCREASE BREAST CANCER RISK ?

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A short title: Estrogens and breast cancer risk

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The etiology of breast cancer is closely linked to the female hormone estrogen, with high lifetime levels being suggested to increase breast cancer risk (*1*). However, there appears to be a great disparity between studies attempting to establish an association between high estrogen levels and breast cancer risk. This disparity becomes smaller by taking into consideration a timing factor, and we therefore propose that estrogens can increase, decrease or have no effect on breast cancer risk, depending on the timing of estrogen exposure. We further propose that timing of estrogenic exposures may play at least as important a role in affecting breast cancer risk than lifetime exposure.

ESTROGENS, CELL PROLIFERATION AND BREAST CANCER RISK

Both normal and malignant breast cells proliferate when exposed to estrogens (*2,3*). Proliferation of malignant cells perhaps explains why estrogens increase breast cancer risk. It is possible that in normal cells, a high rate of cellular proliferation might lead to accumulation of DNA adducts and perhaps eventually mutations. That is because high proliferation rate could give cells less time to repair DNA damage that normally occurs with each round of DNA synthesis. However, the evidence that estrogens would initiate breast cancer is controversial. Estrogen is not a mutagen in the Ames salmonella/microsome direct plate incorporation assay (*4*), suggesting that it may not be genotoxic. There is some evidence that estrogens can actually inhibit mutagenicity of known mutagens in this assay (*5*). In more recent studies estrogens have been reported to be able to induce direct and indirect free radical-mediated DNA damage, genetic instability and mutations in cells in culture and *in vivo* (*6*). Although this would suggest a role for estrogens in cancer initiation, human data have failed to support an apparent association between high estrogen exposure during the time period when breast cancer is most likely to be initiated; i.e., during early adulthood and reproductive years, and increased breast cancer risk. In fact, the opposite may be true, since prepubertal estrogen exposure and pregnancy-linked increase in circulating estrogen levels appear to reduce sporadic breast cancer risk (see below). We therefore propose that only when DNA repair mechanisms are defected, estrogens might initiate breast cancer.

Estrogen receptor α. Estrogen-induced proliferation both in the normal and malignant cells is thought to be mediated by the estrogen receptor (ER) -α. The highest levels of this receptor are present in the least undifferentiated lobules in nulliparous women and the highly proliferative terminal end buds in the rat

mammary gland (7). The ER-α levels become lower and lower by the degree of epithelial differentiation. ER-α levels are remarkably low in the normal 'resting' adult mammary gland, with approximately 7% of cells being ER+ in moderately differentiated human breast lobules (8). Further, in contrast to malignant cells where ER-α is located in the cells that proliferate (8), ER-α and the proliferation-associated marker Ki-67 do not co-localize in either the normal human ($8,7$) or rat breast cells (7). Thus, activation of ER-α may induce cell proliferation indirectly in the normal breast (9).

If ER-α is important in mediating the effects of estrogens in increasing breast cancer risk, its levels should be elevated in women who develop breast cancer. This assumption is supported by findings showing that, during diagnostic surgery, the breast tissue surrounding a malignant tumor expresses higher levels of ER-α than the breast which contains a benign lesion (10). Further, ER-α levels are significantly lower in the breasts of Asian women exhibiting a low breast cancer risk than in the breasts of Caucasian women exhibiting a high breast cancer risk (11).

Since estrogens are known to down-regulate the expression of ER-α ($10,12$), it seems paradoxical that ER-α levels are high in the normal tissue located next to malignant tissue, compared to the normal tissue in the breast which shows no signs of malignancy. In the normal breast (i.e., breast which does not contain a tumor), ER-α content closely follows changes in circulating estrogen levels. For example, breast ER-α content is highest during the follicular phase when estrogen levels are low and lowest during the estrogen peak at the luteal phase (13). These findings suggest that lower circulating estrogen levels during the follicular phase allow ER-α to be up-regulated, while high estrogen levels during the luteal phase down-regulate ER-α. The development of some breast cancers may be associated with a loss of estrogen's ability to down-regulate ER -α (8).

ER-β. In addition to the classical ER-α, at least one other receptor that binds estrogens has been identified: ER-β (14). The discovery of this second estrogen receptor has challenged the classical view of the role of estrogens and estrogen receptors in breast cancer. The ER-β protein is distributed in various estrogen's target tissues, and is detected in both normal and malignant breast cells ($15,16$). Immunohistochenical determination indicates that in the mammary gland, ER-β levels are high from birth to adulthood, throughout pregnancy, lactation and involution (17), while ER-α alpha levels vary greatly from one stage to another, perhaps reflecting their regulation by circulating estrogen levels. It is not known whether circulating estrogen levels affect mammary ER-β protein expression.

Although the specific functions of ER-β in breast are not known, there is some evidence that ER-β may negatively regulate cellular proliferation and have a protective role in normal breast. Hall et al (18) have provided direct proof that ER-β modulates/represses ER-α transcriptional activity in transiently transfected cell lines. They showed that 1) ER-β is a transdominant repressor of ER-α transcriptional activity at subsaturating concentrations of estradiol, and 2) ER-β expression decreases the sensitivity of ER-α expressing cells to estradiol.

There is also indirect evidence supporting the idea of ER-β as an inhibiting mechanism against cellular proliferation. First, ER-β is the predominant ER in the normal rat and human mammary gland and in benign breast disease, but the ratios of ER-α and ER-β gene expression change during carcinogenesis: ER-β mRNA is downregulated and ER-α mRNA is upregulated ($17,19,20$). Indeed, many investigators have found that ER-β mRNA expression in most breast tumors is much lower than ER-α ($20,19,21$). Second, we have found that animals that exhibit a reduced breast cancer risk, have increased ER-β levels in the developing mammary gland (22). In support of these data, Prins et al. (23) showed that reduced ER-β expression correlates with increased risk of prostate cancer in male rats.

ESTROGENS AND INCREASED BREAST CANCER RISK

If estrogens increase breast cancer risk by stimulating the growth of malignant cells, the association between increased risk and estrogens should be seen in women whose breasts have already undergone the first steps of malignant transformation. These women may include women at high familial risk. Mutations in DNA repair genes in high risk families (for example BRCA1 and BRCA2) increase the rate of mutations in other critical breast cancer genes, increasing the risk of events leading to carcinogenesis.

Although very few studies have directly investigated whether estrogenicity increases breast cancer risk in women who have inherited mutated DNA repair genes, there is compelling indirect evidence to suggest so. For example, if one of the BRCA1 alleles is inactivated due to a mutation, estrogens might be more likely to cause genomic instability than if both alleles are functioning normally. Indirect evidence suggests that this is the case, and also that the remaining allele cannot compensate for the loss of the function of the mutated allele. Oral contraceptives stimulate cellular proliferation, and when used prior to first pregnancy, may increase breast cancer risk in BRCA1 carriers (24). However, early oral contraceptive use also increases sporadic breast cancer risk (25). Since oral contraceptives inhibit ovarian estrogen production, which is at least partially compensated with estrogenicity of the pill, it is unclear how oral contraceptives (i.e.; due to high levels of synthetic estrogens or due to lack of ovarian estrogens increase inherited and sporadic breast cancer risk.

Other indicators of estrogenicity have been suggested to increase inherited breast cancer risk. Women possessing germline mutations in BRCA1 are particularly susceptible to breast cancer as a result of pregnancy (26,27). Pregnancy increases circulating estrogen levels by approximately 50-100 -fold. Further, women with a strong family history of breast cancer (approximately 50% of these women are BRCA1 mutation carriers and most of the others carry a mutation in some other tumor suppressor gene) exhibit a 4-fold increase in breast cancer risk, if they had high body mass index (BMI) at the age of 12 (28). High BMI may be indicative of high estrogen exposure, since adipose tissue is an important site of estrogen production.

Evidence also indicates that low estrogen levels reduce inherited breast cancer risk. Smoking reduces breast cancer risk in germline BRCA1 mutation carriers (29). Smokers are reported to have lower circulating estrogen levels than non-smokers, although the association has not been confirmed in all studies (30,31,32). Further, smokers tend to have lower body mass than non-smokers, and thus have less adipose-tissue available to convert estrogens' precursors to active estrogens in their circulation. It has also been found that bilateral prophylactic ovariectomy, which eliminates ovarian estrogen exposure, is associated with a significantly reduced breast cancer risk in women who carry a BRCA1 mutation (33).

These findings suggest that estrogens always increase breast cancer risk in individuals carrying a mutated BRCA1. Consequently, developmental periods characterized by sharp increases in estrogen levels, such as puberty and pregnancy, could pose a particularly high risk for familiar breast cancer initiation and promotion. Our goal is to determine whether some inherited breast cancers could be prevented by maintaining estrogen levels at relatively low, but safe levels at times when ovarian estrogen production starts at puberty or during pregnancy when placental estrogen production is high.

Estrogen exposure has clearly been shown to increase breast cancer risk among some postmenopausal women (34). Their breasts are more likely to have acquired malignant changes than breasts of younger women due to an age-dependent increase in DNA damage and mutations. Estrogen levels are generally higher in postmenopausal women who have developed breast cancer than those who do not (35,34). Obesity also increases postmenopausal breast cancer risk (36), and adipose tissue in obese postmenopausal women is the important source of estrogens. Finally, exposure to estrogens in the form of hormone replacement therapy modesty increases postmenopausal breast cancer risk (37).

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ESTROGENS AND THE PRE-INITIATION OF BREAST CANCER

In utero estrogenic exposures may imprint the mammary gland in a manner that increases its susceptibility to malignant transformation. It has been suggested that the higher the in utero estrogenicity, the higher the subsequent risk of breast cancer (38). For example, breast cancer risk is elevated in women with a high birth weight (39,40), which in turn is strongly related to higher maternal estrogen levels (41). However, the effect of high birth weight has not been confirmed in all studies, and in some studies high birth weight increases only premenopausal breast cancer risk (42). A recent study showed that those twins whose birth weight was high, had a particularly high risk to develop breast cancer (43). In utero estrogenic exposure levels of twins are higher than those of singletons (44), and they are at an increased breast cancer risk (45,46), even without taking into consideration their birthweight (which in fact is often low due to prematurity at birth).

Women who develop premenopausal breast cancer more often may have a genetic predisposition to this disease than women who develop breast cancer after menopause. If high birth weight specifically increases premenopausal breast cancer, women at high familial risk who develop breast cancer could have had increased birth weight. It is to be noted that germline mutation carriers often have a low birthweight (47), perhaps reflecting the role of BRCA1 in fetal growth (48), and therefore even a birthweight considered normal may be associated with increased risk of developing inherited breast cancer.

Consistent with the in utero estrogenicity hypothesis, low fetal estrogenicity appears to be associated with reduced breast cancer risk. For example, daughters whose mothers suffered from pre-eclampsia/eclampsia during pregnancy, which is associated with low circulating estrogen levels, exhibit a significantly lower breast cancer risk (49).

Animal studies indicate that a maternal exposure to an elevated estrogenic environment, as induced by an administration of either estradiol (E2) (50), the synthetic estrogen diethylstilbestrol (DES) (51), the phytoestrogen genistein (52), or through a maternal diet high in n-6 polyunsaturated fatty acids (PUFAs) (53,50), significantly increases breast cancer risk among female offspring.

Recent findings in Asian women, however, appear inconsistent with the in utero estrogenicity hypothesis. Asian women, who have a low breast cancer risk, exhibit significantly higher pregnancy estrogen levels than Caucasian women (54). It is not known why pregnancy estrogen levels are high in Asian women; among non-pregnant women circulating estrogens are 40% lower in Asian than Caucasian women (55). Our animal data suggest that differences in diet between the two ethnic groups may affect pregnancy estrogen levels and perhaps explain why Asian women still have a low breast cancer risk. High dietary intake of soy and fish oils, characteristic of an Asian diet, increase pregnancy estrogen levels in rats, but they either reduce (fish oils) or have no overall effect (soy isolate) on mammary tumorigenesis (our unpublished data). Among Caucasian women (who consume diets low in both soy and fish oils), an association between high in utero estrogenicity and later breast cancer risk could still exist.

Effects of *in utero* estrogen exposure on estrogen receptor α and β. The effects of in utero estrogenic exposures on the target tissue are likely to be mediated at least partly through their interactions with ER proteins. Data obtained in studies in which pregnant rat dams received the synthetic estrogen DES show that total ER content is reduced in both the offspring's mammary glands and the DMBA-induced tumors that arise in these rats (56,57,58). Since ER-β is the main subtype present in the rat mammary gland, it is likely that the reported reduction in total ER content in rats exposed to DES in utero reflects a reduction in ER-β levels. This is supported by findings in male rats. Neonatal estrogenization of male rats with DES increases susceptibility to estrogen-induced carcinogenesis of the urogenital tract. This neonatal estrogenization also increases the expression of ER-α and decreases the expression of ER-β in the adult rat prostate (23). In addition, many investigators have studied changes in the expression of a variety of genes in several target

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tissues and found alterations in several other genes. Taken together, these findings indicate that high fetal estrogenic environment may increase the mammary ER-α/ ER-β ratio, perhaps making the gland more susceptible to malignant transformation.

ESTROGEN EXPOSURE DURING REPRODUCTIVE YEARS AND BREAST CANCER RISK

From an evolutionary standpoint it might not make sense if estrogens were harmful during the years when they are needed for reproductive functions. Breast cancer often occurs late during the reproductive years or after them; i.e., there may not be a selective pressure against the effects of for example *in utero* estrogens on breast cancer risk. Indeed, although life-time estrogen exposure may increase postmenopausal breast cancer risk, the evidence suggesting that estrogen exposure during reproductive years would increase premenopausal breast cancer risk is weak. Below, the effects of circulating estrogen levels, length of menstrual cycle, use of oral and other hormonally-based contraceptives, and different life-style factors, including body weight, diet, exercise, and exposure to environmental estrogens during premenopausal years on breast cancer risk are reviewed briefly. Some data are consistent with the association of reduced estrogenicity in reducing breast cancer risk, while some data suggest that increased estrogen exposure during the early reproductive years reduces breast cancer risk. Other studies suggest no change in risk.

Reduced estrogen levels/activation of ER. Two distinct examples exist relating to the importance of estrogens during premenopausal years in affecting breast cancer risk exist. First, removal of ovarian estrogens by bilateral ovariectomy reduces the risk of developing postmenopausal breast cancer and it is effective as a treatment for existing pre-menopausal disease (*59*). However, unilateral ovariectomy either has no effect, or modestly increases the risk (*60*). The other example is the effectiveness of tamoxifen, an ER antagonist in the human breast, in preventing primary pre- and postmenopausal breast cancer (*61*). Nevertheless, there is no evidence that high estrogen levels would increase premenopausal breast cancer risk.

Circulating estrogen levels. Several studies have investigated whether circulating estrogen levels are associated with premenopausal breast cancer. Among four prospective studies, no significant associations were found between serum estrogens and premenopausal breast cancer risk (*62*). Key et al. summarized the data obtained in 21 human studies, and found that approximately 40% report a reduction in luteal phase estrogen levels in women with breast cancer, and 60% report no change (*63*). All these studies, however, show slightly higher or similar follicular phase estrogen levels in breast cancer cases than in women not diagnosed with breast cancer. Thus, women who will develop breast cancer might exhibit an altered pattern of circulating estrogens. During the follicular phase, when estrogen levels are generally low, high risk women might have modestly elevated levels relative to low risk women. During the luteal phase, however, when estrogen levels are high following two peaks (one peak just prior to ovulation and another smaller one prior to menstruation) in normal women, this hormone might be reduced among high risk women. At present, no explanation can be offered for this altered pattern of estrogen levels during follicular and luteal phases in high risk women.

Polymorphisms in genes that code for metabolizing enzymes for estrogens are known to affect circulating estrogen levels, and therefore are suggested to affect breast cancer risk. These genes include CYP17 and catechol-O-methyltransferase (COMT). CYP17 encodes the rate-limiting step in androgen production: a polymorphism in A2 allele (A2/A2 genotype) is associated with elevated levels of estrogens both in pre- and postmenopausal in women (*64,65*). Nevertheless, the evidence linking this polymorphism to breast cancer risk is controversial (*66*). Recent evidence indicates that A2/A2 genotype may predispose to (inherited?) breast cancer in young women (*67,68*). COMT, in turn, inactivates catechol estrogens and therefore women with low activity allele (LL) have increased circulating estrogen levels. Again, the evidence linking LL polymorphism to increased breast cancer risk is not very strong (*66,69*), and may be associated with breast cancer in young women (*70*). However, some findings indicate that low COMT activity may increase development of postmenopausal breast cancer (*71*), consistent with the idea that high circulating estrogen

levels increase the risk in postmenopausal women.

A recent prospective study in Japanese women showed that serum estradiol levels were increased during the premenopausal years in women who developed postmenopausal breast cancer (72). This finding suggests that, although estrogen levels during the reproductive years do not appear to increase premenopausal breast cancer risk, they might be involved in the etiology of breast cancer which is diagnosed postmenopause.

Menstrual cycle. As indicated above, estrogens peak twice during each normal menstrual cycle: prior to ovulation and prior to menstruation. Thus, if cumulative exposure is an important etiological factor for breast cancer, the higher the number of menstrual cycles a women is exposed to during her lifetime, the higher her risk should be. However, short menstrual cycle length, which would increase the number of cycles in a given time, is not linked to increased breast cancer risk (73) or risk of recurrence (74). Indeed, some studies indicate that a short menstrual cycle length is associated with reduced breast cancer risk (75).

Pregnancy. Circulating estrogen levels are elevated by 50-100 -fold during pregnancy (76). However, it is known that multiple pregnancies provide a strong protective effect against breast cancer (77). Further, women who were younger than 20 years at the time of their first full-term pregnancy exhibit a low breast cancer risk (78). Pregnancy after the age of 30 increases life-time breast cancer risk (78). Pregnancy also induces a short-term increase in risk in women who are 25 or older at the time of their first pregnancy (79,80). In these women, the risk to develop breast cancer can be increased up to 20-fold within the first year after pregnancy (81). The increased risk is estimated to last approximately 5 years following the last full-term pregnancy (82,81), after which the risk returns to the appropriate life-time level.

Both the pregnancy-linked reduction in younger women and increase (either the short-term or the life-long increase) in breast cancer risk in older women may be affected by high pregnancy estrogen levels. A recent animal study in nulliparous rats indicates that a short-term treatment with estradiol and progesterone, at levels mimicking pregnancy, is very effective at protecting the animals from developing mammary tumors induced by a chemical carcinogen (83). Estrogen alone has a similar protective effect, while progesterone alone increases the risk (83). The protective effect may be due to stimulation of ductal branching and extensive formation of more differentiated alveolar lobules in the mammary epithelial tree of the mother (84,85,86,87). These changes also are regulated by various other hormones and growth factors (88,89,90). The differentiated lobules do not give rise to breast tumors (85), which may explain why pregnancy, when it occurs at a young age, protects against breast cancer. In women older than 25, the high pregnancy estrogen levels may, at least for the duration of exposure, enhance the growth of cells that have already undergone the first steps of neoplastic transformation. The breasts of relatively older women may have a higher probability of containing such initiated cells than younger women.

Epidemiological studies suggest that the higher estrogen levels are during pregnancy, the higher is the breast cancer risk. Women who give birth to heavy babies (as discussed high birth weight is associated with elevated pregnancy estrogen levels), are at an increased risk of developing breast cancer (91). Severe nausea and vomiting are linked to both high estrogen levels during pregnancy and a significantly increased risk of breast cancer (92). In addition, women who used the synthetic estrogen diethylstilbestrol (DES) during pregnancy exhibit increased breast cancer risk (93). Conversely, maternal breast cancer risk is reduced in women who suffered from pregnancy-induced hypertension, which is associated with low pregnancy estrogen levels (80). A recent unpublished study by Dr. Richardson (personal communication) provide direct evidence in support of the hypothesis and show that high serum estrone levels during pregnancy are linked to increased breast cancer risk.

Oral contraceptives. Intake of oral contraceptives induces a constant exposure to estrogens. However, oral contraceptives also prevent ovulation and inhibit the two estrogen peaks associated with normal menstrual

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cycling. As a result of preventing ovulation, the circulating levels of estrogens, including E2 and estrone are lower in premenopausal women using oral contraceptives than those not using oral contraceptives (*94*). The possibility that oral contraceptives might affect breast cancer risk, has been investigated in numerous studies (*95*). A pooled analysis of 12 separate studies indicate that women under age 45 who were long-term users, exhibit a 42-45% increase in breast cancer risk (*25*). After age 45, however, the risk of developing breast cancer by oral contraceptive use, is not altered. Thus, prolonged use of oral contraceptives might lead to an increase in risk of breast cancer in young women, but not in women who are 45 years or older. Whether this indicates that either a reduced (a prolonged reduction of ovarian estrogens) or an increased exposure to (synthetic) estrogens is responsible for the increase in risk among premenopausal women, remains to be determined. It is known that oral contraceptives increase cell proliferation in the human breast (*96*). The increased proliferation could occur as a consequence of synthetic estrogen exposure, but also due to down-regulation of a biological factor that normally functions to inhibit proliferation (such as ER-β ?).

Some studies have assessed changes in breast cancer risk in women who used a long-acting injectable contraceptive depot medroxyprogesterone acetate (DMPA), a progestogen. As with oral contraceptives, DMPA prevents ovulation, but it does not provide any exposure to exogenous estrogens. Therefore, ovarian estrogen levels in women with DMPA are always low, not even reaching the levels seen during early follicular phase (*97*). Women using injectable DMPA as a contraceptive exhibited an increased breast cancer risk before age 35 (*98,99*). These findings provide support for the hypothesis that ovarian estrogens in young women protect from breast cancer. A similar mechanism (inhibition of ovulation-linked increase in circulating estrogens) might be responsible for the increase in breast cancer risk in young women taking oral contraceptives. However, it also could be that progestins in both oral and DMPA contraceptives stimulate breast cancer growth.

A high-fat intake. Fat intake is reported to affect circulating estrogen levels. In particular, women who reduce their fat intake exhibit a reduction in serum E2 levels (*100,101,102,103*). While it is not clear whether a high-fat diet can increase circulating estrogens in humans, animal studies suggest that specific dietary fats might be able to do so (*50,104*). Both human and animal data indicate that a high-fat diet may increase ER content in the normal breast or in breast tumors (*56,71*). We found that total ER content was increased by 6-fold in mice fed a diet containing 40% energy versus mice fed only 16% energy from fat (*56*).

The data linking a high-fat intake to breast cancer in women are controversial. Most case-control studies (*105,106,107*) and studies performed using animal models (*108,109*), suggest that a high-fat diet is involved in promoting breast cancer. The majority of cohort studies have failed to find an association between dietary fat intake and breast cancer risk (*110,111*). Nevertheless, a recent pooled analysis suggests that high intake of saturated fats increases the risk of developing breast cancer (*112*).

Most studies have not separately analysed the effects among pre- and postmenopausal women. Interestingly, a study that investigated fat intake and breast cancer risk only in premenopausal women reported a significant reduction in risk by a high fat consumption (*113*). It is possible that dietary fat has different, even opposing effects on breast cancer risk, depending on the timing of exposure. In summary, the existing studies do not strongly support the idea that possible elevation in serum estrogen levels by an adult exposure to a high fat diet would increase premenopausal breast cancer risk.

Body weight. It has consistently been shown that there is an inverse correlation between premenopausal body weight and breast cancer risk (*114,115,116,117*). The correlation is not based on a single end-point; i.e., reduced breast cancer risk is not seen only in the most obese premenopausal women (*118*). A recent study indicates that premenopausal women with a low body mass index (BMI) exhibit a several fold increase in breast cancer risk, while women with the highest BMI exhibit only a non-significant reduction in risk (*119*). A frequently offered explanation for the reverse association between premenopausal breast cancer risk

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and body weight is that obese women might be exposed to lower levels of circulating estrogens, because they may not ovulate. However, women with low BMI also may be anovulatory and thus have low ovarian estrogen levels, but as indicated above, their breast cancer risk is high. Further, women taking oral contraceptives are anovulatory, and they exhibit an elevation in breast cancer risk prior to age 45 (*25*).

BMI does not correlate with available estrogen levels in premenopausal women (*120*). The relatively high frequency of anovulatory women at both extremes of body weight range might contribute to the lack of correlation. Adipose tissue is an important source for estrogens, and it is likely that premenopausal women with high amounts of adipose tissue have higher estrogen levels than lean women, even if both groups are anovulatory. Some metabolites of fat have also been shown to activate the P450 aromatase enzyme that converts testosterone to estrogens (*121,122*). Further, a high fat intake reduces the levels of sex hormone binding protein (*123*), increasing the levels of free estrogens in the blood. Thus, it is highly unlikely that obese women would have reduced breast cancer risk due to reduced exposure to estrogens. Whether premenopausal obesity is linked to reduced breast cancer risk through increases in estrogenicity, remains to be determined.

Exercise. Exercise reduces circulating estrogen levels (*124,125*), but the evidence that exercise might inhibit the risk of developing premenopausal breast cancer, is not compelling (*126,127,128*). A study that found a protective effect showed that exercise reduced breast cancer risk among lean women but exhibited no benefits in heavier women (*127*). It is possible that other factors besides hormones that are associated with exercise, might provide protection for thinner women. These factors could include increased intake of food products, such as fruits and vegetables that reduce breast cancer risk and contain components with apparent anti-carcinogenic properties (*129*). Women with low BMI who do not exercise may be consuming a less healthy diet than lean women who exercise, since exercising tends to go together with a healthy life style.

Environmental estrogens. Several environmental estrogens have been identified, including organochlorine compounds and phytoestrogens. *In vivo* studies (*130*) and studies performed in animal models showing that organochlorine compounds have clear estrogenic properties (*131,132*), has led to the assumption that they might promote breast cancer. However, epidemiological evidence does not clearly support a role for organochlorine compounds in promoting pre- or postmenopausal breast cancer in humans (*133,134,135*). Two studies suggest that pesticide levels in adipose tissue might be lower in cases than controls (*136,137*). Again, higher rather than lower estrogenic activity might be related to reduced premenopausal breast cancer risk.

Other environmental estrogens, such as phytoestrogens, are viewed as compounds that reduce premenopausal breast cancer risk (*138*). Most of these phytoestrogens, including the isoflavone genistein in soy-based food products, act as estrogens *in vitro* and *in vivo* (*139,140,141*). Relatively few epidemiological studies have been conducted to address the link between soy intake and breast cancer risk. Our recent meta-analysis of all existing epidemiological studies indicate that a high soy intake reduces the risk of developing breast cancer only in premenopausal women (*142*). These results support the hypothesis that a slightly increased exposure to estrogens, including those originating from environmental sources, might provide some protection from premenopausal breast cancer.

Alcohol. In contrast to the above suggesting no link between high circulating estrogen levels and increased breast cancer risk, alcohol exposure increases both serum estrogen levels (*143,144,145,146*) and breast cancer risk (*147,148*). The mechanism by which alcohol causes a rapid acute or chronic increase in circulating estrogens might be due to, for example, redistribution of existing endogenous estrogens or increased aromatization of testosterone to E2 (*149*). Alcohol also affects the ER-α. A recent study in human breast cancer cell lines have shown that ethanol stimulates the transcriptional activity of the liganded ER-α, although it does not cause *de novo* activation of ER-α in the absence of the ligand (*150*). It is unlikely that

the increase in ER-α activity could be explained solely by an increase of ER-α protein, since the increase in ER-α activity is much greater than the increase of protein levels (10 fold vs 3 fold, respectively). Perhaps reflecting the association between ER-α and alcohol, clinical findings indicate that alcohol may preferentially increase risk of ER-positive breast cancer, at least in postmenopausal women (*151*).

It should be noted that dietary fat and alcohol appear to have similar effects on circulating estrogen levels (increase) and breast ER content (increase). However, only alcohol has been consistently linked to increased breast cancer risk. It is thus possible that alcohol has many other biological effects besides affecting circulating estrogens that might explain its ability to increase breast cancer risk. For example, alcohol reduces circulating folate levels (*152*). Low folate levels might increase cancer incidence (*153,154*), by mechanisms that are independent from estrogen pathways. Alcohol has also been reported to down-regulate BRCA1, at least *in vitro* (*150*).

Summary. The existing data do not support the idea that high premenopausal estrogen levels would increase the risk of developing sporadic premenopausal breast cancer. This might reflect the mechanisms by which estrogens increase breast cancer risk; i.e., by stimulating the growth of malignant cells. Thus, the breasts of young premenopausal women are not likely to have acquired these cells. Breasts of older premenopausal women might contain malignancies, but due to the relative slow growth of transformed cells, tumors may become detectable only during postmenopausal years. In this latter case, elevated premenopausal estrogen levels might promote the growth of a tumor diagnosed at postmenopause. Results obtained by Kabuto et al. (*72*) are in accordance with this suggestion.

If estrogens can initiate sporadic breast cancer, this may happen in older premenopausal women who have partially lost the function of tumor suppressor genes, for example due to hypermethylation. It might still take years before the tumor becomes detectable; i.e., tumors initiated by estrogens during reproductive years will be diagnosed only after menopause. Thus, the fact that estrogen levels during premenopausal years do not increase premenopausal breast cancer risk does not necessarily implicate that they would not affect breast cancer diagnosed at postmenopausal years

ESTROGENS AND REDUCED BREAST CANCER RISK

The time between birth and puberty may play a critical role in determining later susceptibility to breast cancer. Since epidemiological studies are contradictory regarding the hypothesis that a high-fat diet can increase the risk of developing breast cancer, some investigators have proposed that the childhood or adolescent high-fat intake may be critical (*155,156,157*). This assumption is supported by the established connection between (i) high body fat composition and increased levels of circulating estrogens (adrenal estrogen precursors are aromatized to estrone and further to E2 in adipose tissues) (*158*), (ii) high body fat composition and early menarche onset (*159*), and (iii) early menarche onset and increased breast cancer risk (*160*).

However, the results of several studies indicate that a high-fat diet and high BMI at puberty may be related to a lower, not higher, breast cancer risk. Breast cancer risk is reported to be reduced in women who were heavy, or who consumed a high-fat diet, around the time of puberty (*161,114,162*). Further, one recent study indicates that the heavier a girl is at the age of 7, the lower is her subsequent risk to develop breast cancer (*163*). Our study in a cohort of over 3,477 Finnish women born between 1924-33 in Helsinki confirm this observation and further indicate that a low BMI between the ages of 7 and 15 also significantly increases breast cancer risk (*164*). Thus, the leanest girls at puberty have a higher breast cancer risk, and heaviest girls have a lower risk.

Energy (total caloric intake) restriction is linked to longevity, and believed to reduce breast cancer risk, at

least in animal models (*165*). It also is likely to be associated with reduced circulating estrogen levels. A recent epidemiological study investigated the effect of famine in Netherlands during World War II on breast cancer risk (*166*). At the time the famine occurred, these women were either undergoing adolescent growth spurts and menarche, or were young adults who had not yet given birth to their first child. The results of this study indicate that severe famine in women living in rural areas significantly increased subsequent breast cancer risk, regardless of whether the famine was experienced at the time of puberty or prior to giving birth to the first child. These data are in accordance with the studies indicating that high BMI or a high-fat intake at puberty might reduce subsequent breast cancer risk.

It is not clear whether estrogens are involved in explaining the reduced risk in women that were heavy, or consumed a high-fat diet at puberty. Although high body weight in general is positively correlated with available estrogen levels in postmenopausal women (*167*), this may not occur in young girls and adolescent women (*120*). Results obtained in animal studies support the idea that prepubertal estrogen exposure reduces breast cancer risk. In a study with rats, a daily exposure to 10-40 µg E2 between postnatal days 0 and 30 effectively reduced susceptibility to carcinogen-induced mammary tumorigenesis (*168*). Our data indicate that a shorter exposure at lower E2 concentrations during prepuberty successfully reduces carcinogen-induced mammary tumorigenesis in rats without permanently affecting circulating E2 levels or other reproductive parameters (*22*).

In addition to the findings obtained using E2, it has been investigated whether prepubertal exposure to genistein, a phytoestrogen with estrogenic properties (*169*) present in soy, affects mammary tumorigenesis. Further, prepubertal exposure to the phytoestrogen zearalenone which effectively activates the ER-α (*170*), has also been studied. Exposure to either of these two phytoestrogens between postnatal days 7 and 20 effectively reduces carcinogen-induced mammary tumor incidence (*171,172*).

Effects on estrogen receptors. We have determined the concentrations of ER-α and ER-ß by western blot in the mammary glands of rats that were exposed to estradiol during prepuberty. The results indicated that mammary ER-ß levels were increased by 2-fold in the 8- and 16-week-old rats exposed to E2 during prepuberty (*22*). Thus, prepubertal estrogenic exposures might reduce breast cancer risk by up-regulating ER-β in the mammary gland.

WHY TIMING OF ESTROGENIC EXPOSURES DETERMINES THEIR EFFECT ON BREAST CANCER RISK?

Estrogens are required for the development and function of many tissues. Estrogens are of critical importance in bone development, and the maintenance of bone density (*173,174*), and participate in maintaining a healthy cardiovascular system (*175,176*). Estrogens are also important in regulating mood (*177*) and are closely involved in establishing, maintaining and repairing neuronal connections (*178,179,180*). Reproductive functions and normal mammary gland development are also dependent upon estrogens. Since estrogens affect several important and diverse functions, it would be surprising if the adverse effects of estrogens (i.e., their potential to increase breast cancer risk) would dominate over their beneficial effects on normal tissues. We have proposed that a complementary system exists in parallel with estrogens that protect tissues from the adverse (potential for increased DNA damage) effects of estrogens. This system is likely to be composed of genes that help to maintain genomic stability and repair estrogen-induced DNA damage. Tumor suppressor genes are ideal candidates to serve as balancing the potential adverse effects of estrogens. We are currently testing the hypothesis that pubertal estrogenic exposures up-regulate BRCA1 to protect the breast from malignant transformation.

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Cadmium mimics the effects of estrogen *in vivo* in the uterus and mammary gland

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ABSTRACT

Previous studies demonstrate that the heavy metal cadmium activates estrogen receptor-alpha (ERa) in breast cancer cells by forming a high affinity complex with the hormone binding domain of the receptor. The present study shows that *in vivo* exposure to an environmentally relevant dose of cadmium has estrogen like activity in target organs of ovariectomized animals or following exposure *in utero*. Similar to estrogens, exposure of ovariectomized animals to a single dose of cadmium (5 ug/kg body weight) resulted in a 1.9-fold increase in uterine wet weight that was blocked by the antiestrogen ICI-182,780. Histological examination of the uterus revealed that the increase in weight was accompanied by a proliferative response of the endometrium. Exposure of ovariectomized animals to the metal also promoted the growth and differentiation of the mammary gland that was blocked by the antiestrogen. In animals treated with cadmium, there was an increase in side branching of the mammary ducts and in the number of alveolar buds. Immunocytochemical analysis demonstrated production of casein and whey acidic protein in the ducts suggesting that the metal induces a secretory differentiation of the mammary gland. Exposure *in utero* to cadmium also mimicked *in utero* exposure to estradiol. Female animals exposed to the metal, via pregnant dams, had an earlier onset of puberty and altered mammary gland development. Taken together, these data provide strong evidence that environmentally relevant doses of cadmium have estrogen like effects *in vivo*.

INTRODUCTION

Estrogens are a family of steroidal hormones that are synthesized in a variety of tissues but are produced primarily in the ovaries during reproductive years. One of the main functions of estrogens is to promote the growth and differentiation of the sexual organs and other tissues related to reproduction. The disruption of the reproductive system of male and female animals in the wild has been attributed to exposure to environmental contaminants which mimic the effects of estradiol. It has been suggested that the high incidence of hormone related cancers and diseases in the Western world is also due to the presence of environmental estrogens. In fact, a number of chemicals in the environment demonstrate estrogen like activity when tested in biological systems [1].

The biological effects of estrogens are mediated by estrogen receptors (ER) alpha and beta and molecules that bind to and activate these receptors may pose a risk to health. Data presented in this paper and in previously published studies from our laboratory [2,3] demonstrate that the heavy metal, cadmium, possesses estrogenic activity and, therefore, is a candidate nonsteroidal environmental disrupter. In the estrogen responsive cell line, MCF-7, exposure to cadmium mimics the effects of estradiol on cell proliferation and gene expression. It increases cell growth and the steady state amounts of progesterone receptor, pS2, and cathepsin D and decreases the steady state concentration of ERa [2]. The changes in gene expression are due to activation of ERa by the metal; this activation is blocked by an antiestrogen. More recent work demonstrates that ERa activation occurs as the result of a high affinity interaction of cadmium with the hormone binding domain of the receptor [3]. The goal of the present study was to determine whether exposure to a dose of cadmium, similar to the amount present in the environment, mimics the effects of estradiol in

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estrogen target organs following ovariectomy or *in utero* exposure. Exposure of ovariectomized animals to cadmium was found to increase uterine wet weight, promote mammary gland growth, and induce hormone regulated genes. *In utero* exposure to the metal also demonstrated estrogen like effects on the reproductive system and mammary glands. These results provide additional evidence that environmentally relevant amounts of cadmium have potent estrogen like activities.

RESULTS

Effects of cadmium on uterine wet weight in ovariectomized rats

To determine whether in vivo exposure to a low, environmentally relevant dose of cadmium demonstrates estrogen like activity in target organs, animals were given cadmium at a dose of 5 ug/kg body weight (approximately 27 nmol/kg). Prior to treatment, female Sprague-Dawely rats were ovariectomized on postnatal day 28 and the uterus was allowed to involute for three weeks. The animals received a single intraperitoneal (i.p.) dose of cadmium. As a positive control, animals received a pellet of estradiol releasing 60 ug/kg/day. Four days after treatment, uterine wet weight was measured (Table 1). As expected, there was a 3.8-fold increase in uterine wet weight in the estradiol treated animals. In the cadmium treated animals, there was a 1.9-fold increase in uterine wet weight. The metal induced increase in weight was blocked by the antiestrogen ICI-182,780 suggesting that the effects of cadmium are mediated by the estrogen receptor. A similar increase in uterine wet weight was also observed when the animals were given a single i.p. dose of cadmium of 10 ug/kg (1.7-fold increase) or when the animals were ovariectomized on day 40 (1.8 fold increase). Treatment with cadmium had no effect on whole body weight (Table 1) suggesting that the dose of cadmium was not toxic.

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Histological examination demonstrated that the increase in uterine wet weight was due to a mitogenic response and not a toxic response to the metal (Figure 1). In the ovariectomized animals, the endometrial lining was flat to cuboidal. No vacuolation or stromal inflammation was observed. Further, no mitoses were observed in either the endometrial or stromal cells. In the estradiol treated animals, the endometrial lining showed epithelial hyperplasia and hypertrophy. The endometrial cells were taller and had abundant granular cytoplasm. The surrounding stroma was hypercellular and infiltrated by numerous eosinophils. Cadmium treated animals also showed hyperplasia and hypertrophy. In addition, the cells demonstrated abundant subnuclear and supranuclear vacuolation. The stroma was more cellular in the metal treated animals than in the ovariectomized animals but less cellular than in the animals treated with estradiol. No stromal inflammatory infiltrate was noted in the cadmium treated animals. Both estradiol and cadmium treated animals showed rare mitoses in the endometrial cells. No evidence of cadmium toxicity was observed upon histological examination of the liver and kidney, organs sensitive to the toxic effects of the metal (data not shown). Taken together, these data suggest that environmentally relevant doses of cadmium induce an estrogen like response in the uterus.

Effects of cadmium on mammary gland development in ovariectomized rats

To determine whether exposure to environmental amounts of cadmium also influenced mammary gland development, animals were again ovariectomized at 28 days of age and allowed to rest for 3 weeks prior to treatment with either cadmium or estradiol. Animals received either a pellet of estradiol, 60 ug/kg/day, or a single i.p. injection of cadmium, 5 ug/kg body weight. The antiestrogen, ICI-182,780, was administered i.p. at a dose of 500 ug/kg/day. In this study, the

mammary gland was examined on days 4 and 14 after estradiol or cadmium (Figure 2). Epithelial density in the midregion of the fourth inguinal gland was determined using the NIH Image program (Table 2). In the ovariectomized control animals, the mammary gland consisted of a simple ductal network with low epithelial density. In the estradiol treated animals, the density of the gland increased. Following exposure to the hormone, there was a 50% increase in epithelial density by day 4 that was sustained by day 14. The increase in epithelial density was due to an increase in mammary ducts and an increase in abundance of secretory lobuloalveolar structures. In animals exposed to cadmium, there was also a 50% increase in epithelial density by day 4 and a 30% increase on day 14. The metal induced increase in epithelial density was due to an increase in quaternary branching of the ducts and an increase in lobuloalveolar structures. In animals treated with the antiestrogen, the epithelial density was not significantly different from ovariectomized animals. There were less secretory structures in the gland on day 4 with a more pronounced effect observed on day 14. Treatment of animals with the antiestrogen also blocked the effects of cadmium on epithelial density and secretory structures suggesting that the response of the mammary gland to cadmium is mediated by the estrogen receptor.

To determine whether cadmium induced a secretory differentiation of the mammary gland, the effects of cadmium on the expression of casein (Figure 3) and whey acidic protein (data not shown) were examined on day 14. In control animals, the mammary gland was devoid of casein, whereas, animals treated with estradiol for 14 days synthesized significant amounts of the protein. Casein was found in the ductal lumen, alveolar cells, and alveolus. Interestingly, animals exposed to a single dose of cadmium also synthesized significant amounts of casein. The protein was localized in luminal cells, alveolar cells, alveolus, and ductal lumen with most of the casein

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immunolocalized in the cytoplasm. Expression of whey acidic protein was also detected but was not as abundant (data not shown). The ability of cadmium to induce the synthesis of both casein and whey acidic protein demonstrates that the metal induces milk protein synthesis in the mammary gland.

The amount of cadmium in the uteri and mammary gland was determined using anodic stripping voltammetry, an electrochemical technique which offers detection limits in the sub-part-per-billion range [4]. Cadmium was not detected in the organs of control animals but was detectable in most organs of the metal treated animals. However, the amount of cadmium was too low to accurately quantitate. When detectable in the uteri or mammary gland, the amount of the metal was approximately 10^{-2} picograms/gram tissue (i.e., 10^{-5} parts-per-billion).

Effects of *in utero* exposure to cadmium on the development in female offspring

The estrogenic effects of cadmium were also assessed following *in utero* exposure to the metal. It is well documented that *in utero* exposure to estrogens and estrogen like substances causes early onset of puberty, as measured by vaginal opening [5,6], and alters mammary gland development [5,7,8]. In this study, pregnant animals were given two injections of cadmium i.p. at a dose of either 0.5 or 5 ug/kg body weight on day 12 and 17 of gestation. Two days after the offspring were born, the female animals were cross-fostered with a lactating dam and culled to 10 female pups per litter. The female offspring were weaned on postnatal day 22. Cadmium did not alter pregnancy weight gain, the number of pups per litter, or birth weights (data not shown). However, on postnatal day 35, female offspring exposed to the lower dose of cadmium had significantly increased body weights (135.8 +/- 2.4 gm, mean +/- SEM) compared to control female

offspring (120 +/- 2.6 gm, F(2,15) = 13.8, p < 0.001). *In utero* exposure to estrogenic compounds has been shown to induce a temporary increase in body weight at this age [9]. Although exposure of ovariectomized animals to estrogen results in an increase in uterine wet weight, *in utero* exposure to low doses of estrogens does not alter uterine weight [5]. Consistent with this observation, there was no difference in uterine wet weight (either crude or adjusted for body weight) between control animals and animals exposed to cadmium *in utero* (data not shown). However, similar to the early onset of puberty following *in utero* exposure to estrogens [5,6], *in utero* exposure to cadmium resulted in the earlier onset of vaginal opening in female offspring (Figure 4). In control animals, vaginal opening occurred on average on day 30.6 +/- 0.6, whereas, vaginal opening occurred on day 27.2 +/- 1.1 (p < 0.05) and on day 26.7 +/- 1.1 (p < 0.05) in animals exposed to cadmium doses of 0.5 and 5 ug/kg, respectively.

The effects of *in utero* exposure to cadmium on mammary gland development were assessed on postnatal day 35 during the rapid growth phase of the gland. Previous studies have shown that perinatal exposure to estrogenic compounds increases the parenchymal area of the mammary gland and the number of terminal end buds and decreases the number of alveolar buds [5]. Similar estrogen like effects on the mammary gland were also observed in animals exposed to cadmium *in utero*. The mammary epithelial area was significantly larger, 70.7 +/- 5.2 and 66.5 +/- 7.7 (mean +/- SEM) in animals exposed to cadmium doses of 0.5 and 5 ug/kg, respectively, compared to 45.5 +/- 4.2 in control animals (Figure 5A). The mammary glands of animals exposed to the lower dose of cadmium also contained significantly more terminal end buds, 12.5 +/- 1.0, than control animals which contained 9.4 +/- 0.2 (Figure 5B). Similar to *in utero* estrogen exposure, both doses of cadmium reduced the number of alveolar buds in the mammary gland from

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15.0 +/- 3.9 in control animals to approximately 7.5 +/- 1.5 in animals exposed to the metal (Figure 5C). The ability of cadmium to mimic the *in utero* effects of estrogens provides additional support that environmentally relevant doses of the metal have potent estrogen like activities.

DISCUSSION

Cadmium is a transition metal with no known physiological function. To date, most animal studies have examined the toxic and carcinogenic effects of the metal and have employed cadmium doses in the range of 1 to 5 mg/kg (approximately 5 to 25 umol/kg). Exposure to these doses of the metal causes pulmonary adenocarcinomas and sarcomas at the site of injection (reviewed in [10,11]). In male animals, cadmium also induces necrosis of the testis followed by proliferation and formation of testicular tumors (reviewed in [10,11]). In female animals, cadmium induces necrosis of the ovaries with no subsequent formation of ovarian tumors [12,13]. In contrast to previous toxicological studies, our goal was to determine whether lower doses of the metal have estrogen like activity in target organs. Earlier studies from our laboratory demonstrate that cadmium acts functionally like steroidal estrogens in breast cancer cells as a result of its ability to form a high affinity complex with the hormone binding domain of ER*a*. The results of this study show that cadmium also has potent estrogen like activities *in vivo*. There was a significant proliferative response in the uterus and mammary gland of ovariectomized animals administered a single environmentally relevant dose of the metal. Exposure to cadmium resulted in an increase in uterine wet weight that was accompanied by estrogen like changes in proliferation in the organ. Exposure to the metal also affected the mammary gland. There was an increase in branching of the mammary ducts and in the number of alveolar buds. Secretory differentiation of the gland was also seen. The

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ability of an antiestrogen to block the cadmium induced changes suggests that the effects of the metal are mediated by the estrogen receptor. *In utero* exposure to the metal also mimicked the effects of *in utero* exposure to estrogens. Female offspring displayed altered mammary gland development and an early onset of puberty.

Cadmium is used principally in galvanizing and electroplating, in batteries, in electrical conductors, in the manufacture of pigments, plastics stabilizers, and, most importantly, in the stabilization of phosphate fertilizers [14]. Although cadmium is widely distributed in the earth, the primary exposure to cadmium in the environment is through contamination from metal smelting operations [15]. In the 1980s, the total atmospheric emissions of cadmium were estimated to be about 1.4 million pounds annually. In the year 2000, it is estimated that cadmium usage in the United States will be 36 million pounds. The level of cadmium contamination in the air and soil is generally reflected in the level of cadmium in streams and rivers. However, water may contain cadmium as a result of leaching from the soil and the dissolution of cadmium from underlying geologic formations, especially in areas where soft, acidic waters are common [14].

Non-occupational exposure to cadmium occurs primarily through dietary sources, cigarette smoking, and, to a lesser degree, drinking water [16,17]. In the United States, dietary studies in the late 1970s and early 1980s found that potato food groups and grain or cereal products accounted for the largest portion of cadmium intake in the adult male, contributing 24 and 36 percent of exposure, respectively. Fluids, which include drinking water, accounted for 3.2% of cadmium intake. It was estimated that the average daily intake of cadmium in the adult male is 22.63 ug/day (approximately 0.3 ug/kg/day). Cigarette smoke is also an important source of human exposure, reflecting the high efficiency of pulmonary absorption of inhaled cadmium [18,19]. Cadmium intake

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from one pack of cigarettes per day is estimated to be 2-4 ug. In general, the amount of cadmium found in newborns is negligible, but by age 30, the body burden may reach 30 mg. The estimated biological half-life of cadmium ranges from 10 to 30 years [19] which may account for the significant accumulation of the metal in the body. In nonsmokers, the kidney concentration of cadmium is approximately 15-20 ug/gm wet weight, while in smokers, the concentration doubles to 30-40 ug/gm wet weight. High concentrations of cadmium are also present in breast fat of healthy women and breast cancer patients (20-30 ug/gm) [20]. This is in stark contrast to the amount found in the present study. The mammary glands of the animals exposed to a single, environmentally relevant amount of cadmium contained approximately 10^{-2} pg/gm tissue. Nevertheless, the metal had profound effects on the growth and development of the gland suggesting that exposure to cadmium may be a potential risk factor for breast cancer.

Epidemiological studies suggest that endocrine factors play an important underlying role in the etiology of breast cancer. Prominent risk factors related to endocrine status include age at menarche and menopause and age at first full-term pregnancy [21]. Menarche at age 11 or earlier compared to age 15 or greater confers an approximately 50% increase in breast cancer risk depending on the age at diagnosis [22,23]. This increase in risk is thought to reflect the larger lifetime number of normal menstrual cycles [24]. A twofold increase in risk is also associated with increasing age at first full-term pregnancy with perhaps a slightly greater risk for women giving birth at age 30 or greater compared to nulliparous women [25]. Oral contraceptives and replacement estrogens account for a small increase in risk in specific subgroups of women [26,27]. On the other hand, a decrease in risk is associated with bilateral ovariectomy and earlier age at menopause reflecting the decrease in levels of estrogen [28,29]. The primacy of estrogens in the epidemiology of the disease is

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due to the hormonal control of proliferation of breast cells [30,31]. Environmental exposures which mimic the effects of estrogens may be potential risk factors for breast cancer. However, the only epidemiologic study to suggest a link between cadmium exposure and breast cancer risk [32] is a hypothesis-generating case-control study based on death certificates coded for occupation and industry. After excluding homemakers, a job exposure matrix was used to estimate the probability of risk for occupational exposure to cadmium and found an odds ratio of 1.07-1.13 among white women and 1.5-2.3 among black women. Further studies are required to substantiate these findings.

Previously, we have demonstrated that cadmium has potent estrogen like activity *in vitro* [2,3]. The metal forms a high affinity complex with ERa and, thereby, activates the receptor in cultured cells. The data presented in this study provide strong evidence that cadmium is also a potent activator of the estrogen receptor *in vivo*. Exposure to an environmentally relevant dose of the metal mimics the effects of estradiol in target organs of ovariectomized animals or following *in utero* exposure. The ability of environmental amounts of cadmium to mimic the effects of estradiol suggest that the metal is a potent endocrine disrupter.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Harlan, Indianapolis, IN) were employed in this study. Animals were ovariectomized by the vendor at 28 or 40 days of age and the animals were allowed to recover for 2 to 3 weeks prior to treatment with cadmium, estradiol, or the antiestrogen, ICI-182780. Animals were housed under a 12 hour light-dark cycle. Cadmium chloride (Sigma, St.

Louis, MO) was dissolved in sterile phosphate buffered saline and administered intraperitoneal. An estradiol 30 day release pellet (Innovative Research of America, Sarasota, FL) was implanted subcutaneously. The antiestrogen, ICI-182,780 (Tocris, Baldwin, MO), was dissolved in peanut oil and given intraperitoneal at a dose of 500 ug/kg/day. To study the effects of the metal on the uterus, animals were given a single i.p. injection cadmium at a dose of 5 ug/kg or 10 ug/kg body weight (approximately 27 or 54 nmol/kg). Animals were euthanized 4 days later and the effects of cadmium on uterine wet weight and histology were examined. To study the effects of cadmium on mammary gland development, the animals were ovariectomized as above. Animals received a single i.p. injection of cadmium (5 ug/kg) on day 1 and the effects of the metal on mammary gland development and on the synthesis of casein and whey acidic protein were examined on days 4 or 14.

Pregnant CD-1 mice (NCI, Fredrick, MD) were obtained on day 10 of gestation. The animals were individually housed in standard plexiglass cages under a 12 hour light-dark cycle. Pregnant rats were injected i.p. with either 0.5 or 5 ug/kg cadmium or vehicle on day 12 and day 17 of gestation. Two days after the birth, the male offspring were sacrificed and the female offspring were cross-fostered. Ten female pups were housed with a lactating dam. The female offspring were weaned on postnatal day 22 and, thereafter, housed in groups of 3 to 5 animals. Vaginal opening was determined blind to treatment. Data were analyzed by one-way ANOVA followed by Fisher LSD method.

Mammary whole mounts

Mammary whole mounts were prepared as follow. After the animals were sacrificed, the

inguinal mammary glands were removed and fixed in ethanol-glacial acetic acid (3:1, v/v). The glands were then stained with carmine alum, dehydrated through graded alcohols, and then cleared with xylenes prior to coverslipping with permount. Mammary epithelial density in the rats was calculated by outlining a 2.05 cm^2 section of the midregion of the fourth inguinal gland using NIH image. In all treatments, the amount of epithelial density was inversely proportional to light intensity. Images were then transferred to MacIntosh Power Station and processed using Abode Photoshop Software. In the mouse mammary gland, the terminal end buds and alveolar buds were counted and the area was measured.

Immunocytochemistry

For immunohistochemical examination of casein synthesis in the mammary gland, samples were fixed in 10% formalin and dehydrated in ethanol (65 to 100%). The samples were then cleared in xylene, infiltrated with paraffin, and embedded in a block. Five micron sections were obtained and visualized with a routine hematoxylin and eosin stain. Additional sections were processed for immunohistochemical localization of casein using a horseradish peroxidase technique. Briefly, tissue sections were deparaffinized, washed in Tris buffered saline, pH 7.6 (TBS) containing 0.6% Tween-20 (TBS-T, Bio-Rad, Hercules, CA), and then treated with 3% hydrogen peroxide in deionized water to remove any endogenous peroxidase. After washing in TBS-T, tissue sections were covered with 10% bovine serum albumin in TBS-T for 1 hour. After washing in TBS-T, excess buffer was removed and tissue sections were incubated for 2 hours at room temperature with an anti-mouse pan-casein antibody (generous gift of G. Smith, NCI, NIH, Bethesda, MD) diluted 1:1000 or anti-mouse whey acidic protein (generous gift from L.

Henninghausen, NIDDK, NIH, Bethesda, MD). After this incubation period, tissues were washed in TBS-T three times for 5 minutes each. Peroxidase staining was performed using mouse Vectastain kit ABS (Vector Laboratories, Burlingame, CA).

Anodic stripping voltammetry assay

The amount of cadmium in the tissue was determined using an anodic stripping voltammetry assay. In this assay, the tissue were ashed in a muffle furnace at 450^0 C for 12 to 24 hours. The ash was then digested with trace metal grade nitric acid (Fischer, Pittsburgh, PA), the nitric acid was removed by evaporation to near dryness, and the sample was dissolved in distilled water containing mercuric nitrate. In the anodic stripping voltammetry assay, the sample was placed in an electrochemical cell containing a working electrode made of glassy carbon, a platinum counter electrode, and a pseudoreference electrode constructed of platinum. The cadmium ion and other metal analytes were preconcentrated as cadmium in the mercury film that forms on the working electrode. After the preconcentration step, the working electrode voltage was scanned in the positive direction at 50 to 100 mV per second. Current measurements during this scan yielded peaks corresponding to the reoxidation of the metal analytes in the mercury film. Oxidative current peaks corresponding to cadmium were compared with standard curves to determine the cadmium content in the tissue samples. The EG&G PARC 270 Electrochemical Analysis Software was employed for data analysis and manipulation.

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FIGURE LEGENDS

Table 1. Effects of cadmium on uterine wet weights in ovariectomized animals.

Female Sprague-Dawley rats were ovariectomized at 28 days of age and the uterus was allowed to involute for 3 weeks prior to treatment with a single i.p. injection of cadmium (5 ug/kg body weight), estradiol (60 ug/kg/day), or the antiestrogen ICI-182,780 (500 ug/kg/day). Animals were sacrificed 4 days later and the effects of cadmium on uterine wet weight were measured. Data are the mean value (+/- SEM).

Figure 1. Histological effects of cadmium in the uteri of ovariectomized animals.

Female Sprague-Dawley rats were ovariectomized at 28 days of age and the uterus was allowed to involute for 3 weeks prior to treatment with a single i.p. injection of cadmium (5 ug/kg body weight), estradiol (60 ug/kg/day), or the antiestrogen ICI-182,780 (500 ug/kg/day). Animals were sacrificed 4 days later and the uteri were dissected, formalin fixed, and paraffin embedded. Sections were stained with hematoxylin and eosin (200X).

Figure 2. Effects of cadmium on mammary gland development in ovariectomized animals.

Female Sprague-Dawley rats were ovariectomized at 28 days of age and the animals were allowed to rest for 3 weeks prior to treatment. Animals were given estradiol (60 ug/kg/day) or a single i.p. injection of cadmium (5 ug/kg body weight) and the effects on mammary gland development were examined on day 4 and 14. Whole mounts of the fourth inguinal gland were prepared and the glands were stained with carmine alum.

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Figure 3. Effects of cadmium on the histology and synthesis of casein in the mammary gland.

Female Sprague-Dawley rats were ovariectomized at 28 days of age and the animals were allowed to rest for 3 weeks prior to treatment. Animals were administered either estradiol (60 ug/kg/day) or a single i.p. injection of cadmium (5 ug/kg body weight) and the effects on mammary gland histology and casein production were examined on day 14. Sections of gland were visualized as described in materials and methods.

Figure 4. Effect of *in utero* exposure to cadmium on vaginal opening in female offspring.

Pregnant CD-1 mice were given cadmium i.p. at a dose of either 0.5 or 5 ug/kg on days 12 and 17 of gestation. Two days after birth, female offspring were cross-fostered and housed in litters containing 10 female pups. Vaginal opening was monitored as a measure of the onset of puberty. Data were analyzed by one way ANOVA: $F(2,22) = 5.8$, $p < 0.001$. Both doses of cadmium advanced puberty onset ($p < 0.05$).

Figure 5. Effect of *in utero* exposure to cadmium on the mammary gland in female offspring.

A. Effects of *in utero* exposure to cadmium on mammary epithelial area.

B. Effects of *in utero* exposure to the metal on the number of terminal end buds.

C. Effects of *in utero* exposure on the number of alveolar buds.

Pregnant CD-1 mice were given cadmium i.p. at a dose of either 0.5 or 5 ug/kg on days 12

and 17 of gestation. Two days after birth, female offspring were cross-fostered. The effects of in utero exposure to cadmium on mammary gland development were assessed on postnatal day 35. Values represent the mean of six animals per group +/- SEM. Data were analyzed by one way ANOVA: epithelial area, $F(2,15) = 5.2$, $p < 0.02$; TEB, $F(2,14) = 11.6$, $p < 0.001$; AB, $F(2,14) = 2.7$, $p < 0.01$. TEBs, terminal end buds; ABs, alveolar buds; *, $p < 0.05$ significantly different from controls.

Table 1. Effects of cadmium on uterine wet weight in ovariectomized animals

	uterine wet weight, grams	fold increase	body weight, grams
Control	0.075 (+/-0.0069, n=17)		187 (n=9)
Cadmium	0.141* (+/-0.0111, n=21)	1.9	189 (n=12)
ICI-182,780	0.048 (+/-0.0027, n=13)	0.64	182 (n=10)
Cadmium + ICI-182,780	0.046 (+/-0.0035, n=11)	0.61	182 (n=8)
Estradiol	0.284** (+/-0.0168, n=22)	3.8	172 (n=10)

Female Sprague-Dawley rats were ovariectomized on day 28 and the uterus was allowed to involute for 3 weeks prior to treatment with cadmium (5ug/kg body weight), estradiol (60 ug/kg/day), or the antiestrogen ICI-182,780 (500 ug/kg/day). Animals were sacrificed 4 days later and the effects of cadmium on uterine wet weight were measured. Data are the mean value (+/- SEM). *, p=0.0001 compared to control; **, p<0.0001 compared to control.

Table 2. Effects of cadmium on mammary gland density

	Day 4	Day 14
Control	54.3 (+/- 2.5, n = 17)	75.4 (+/-1.9, n = 9)
Cadmium	82.8* (+/- 4.0, n = 20)	99.8* (+/- 2.9, n = 14)
ICI-182,780	69.7 (+/- 3.7, n = 8)	72.7 (+/- 2.2, n = 8)
Cadmium + ICI-182,780	69.0 (+/- 5.4, n = 8)	72.2 (+/- 2.8, n = 8)
Estradiol	84.1* (+/- 3.2, n = 20)	112.6* (+/- 6.9, n = 11)

Female Sprague-Dawley rats were ovariectomized at 28 days of age and the animals were allowed to rest for 3 weeks prior to treatment. Animals were given estradiol (60 ug/kg/day) or a single i.p. injection of cadmium (5 ug/kg body weight) and the effects on mammary gland development were examined on day 4 and 14. Whole mounts of the fourth inguinal gland were prepared and the epithelial density was measured using NIH image. Data were analyzed by one-way ANOVA: $F_{(4,68)} = 12.41$, $p < 0.001$ for day 4; $F_{(4,46)} = 20.73$, $p < 0.001$ for day 14. Values are the mean (+/- SEM). Significantly different from controls: *, $p < 0.05$.

Control
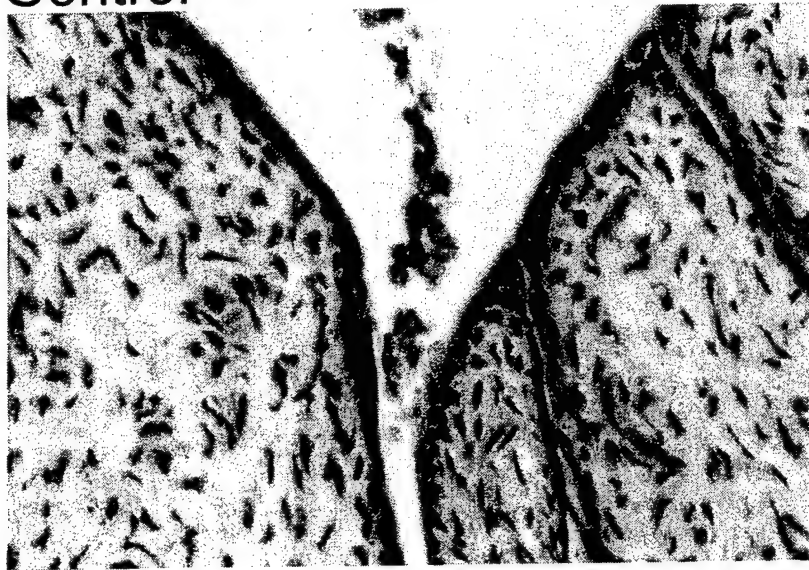
Estradiol
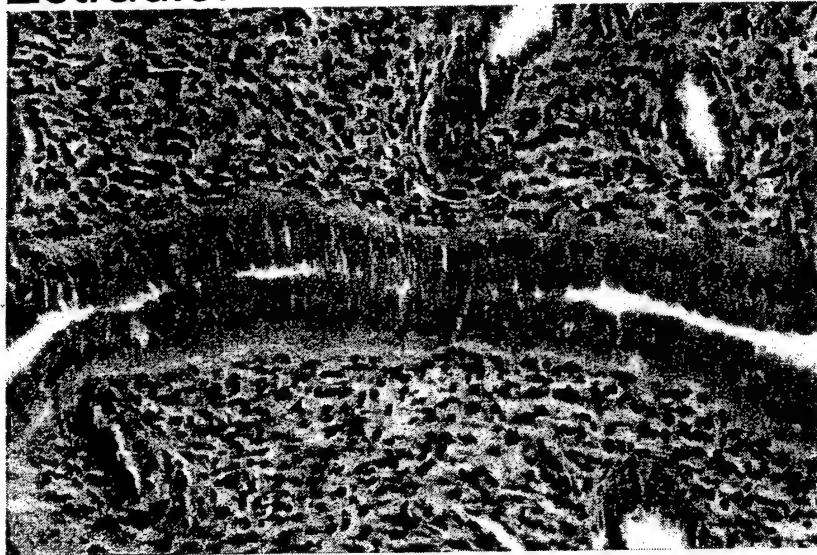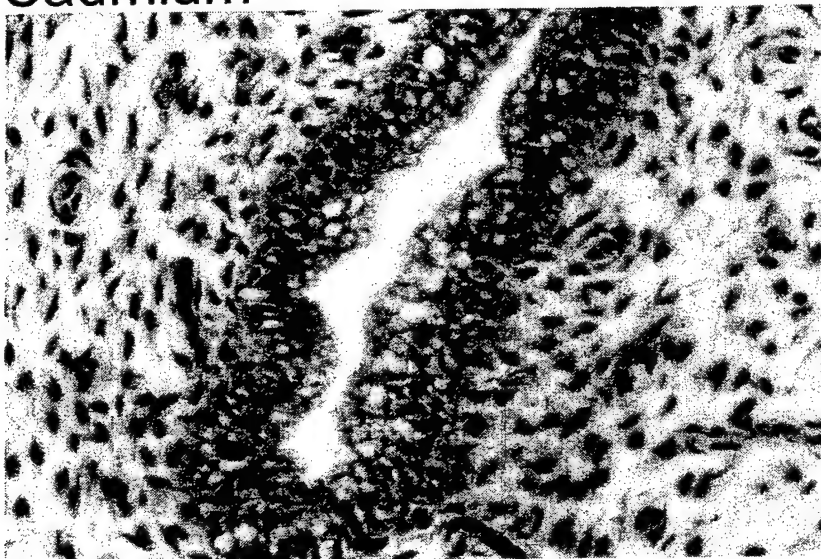
Cadmium

Figure 1

Control   Estradiol   Cadmium
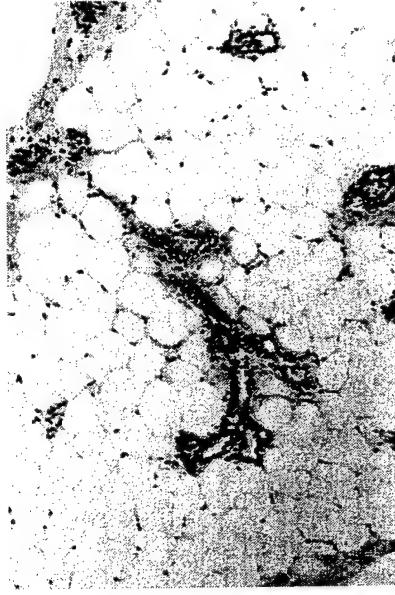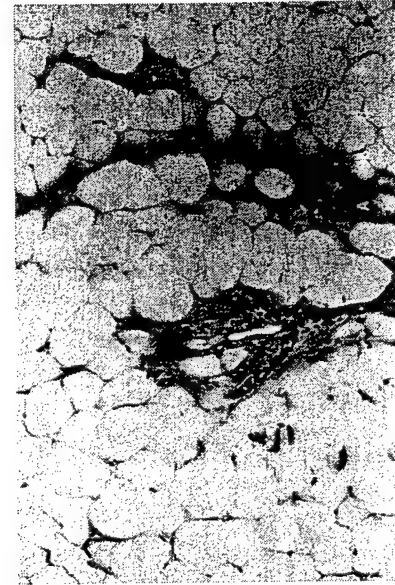
Casein

Figure 3
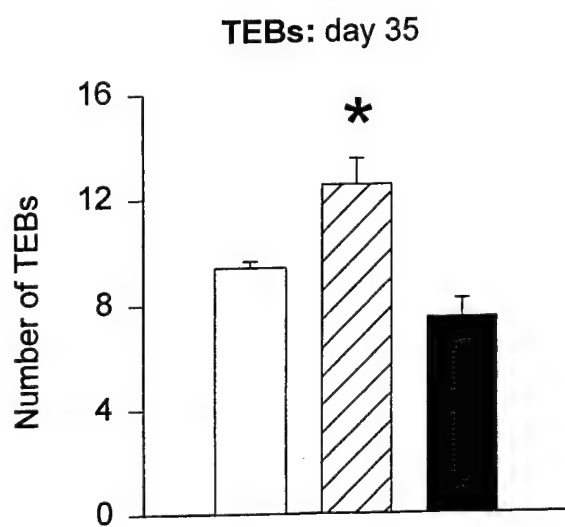
In utero cadmium exposure: vaginal opening

Figure 4

Epithelial area: day 35

Figure 5A

TEBs: day 35

Alveolar buds: day 35

Figures 5 B and C

Review
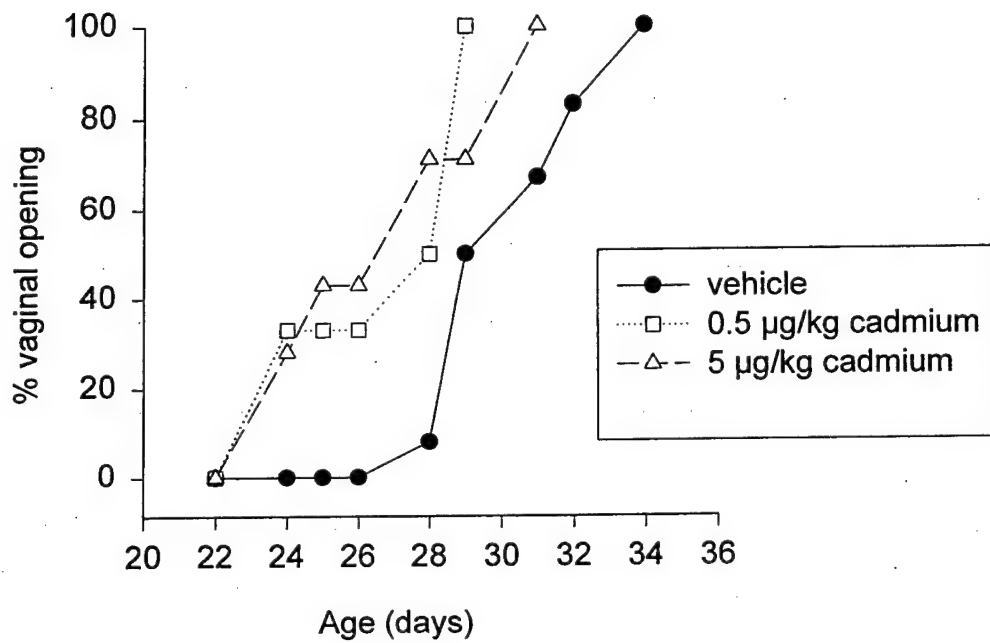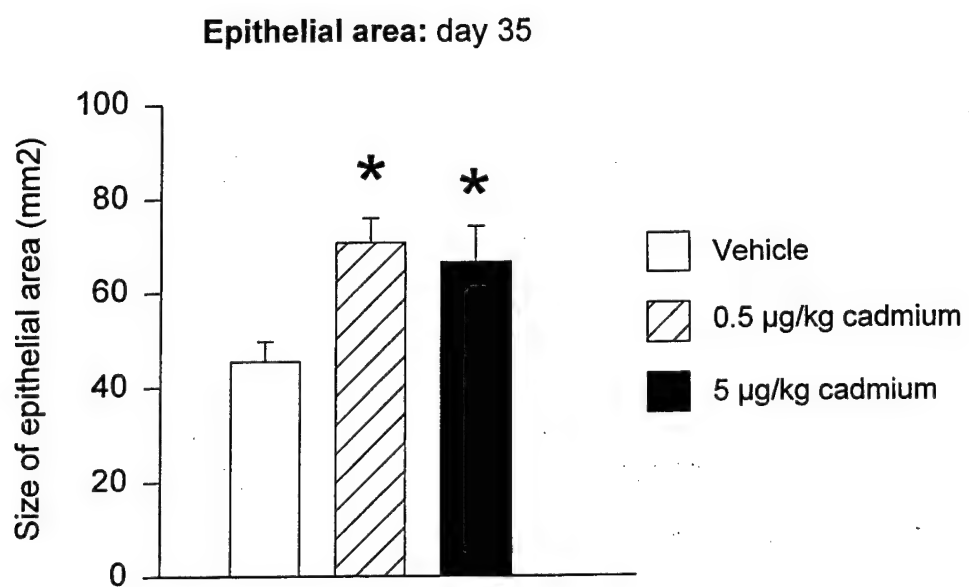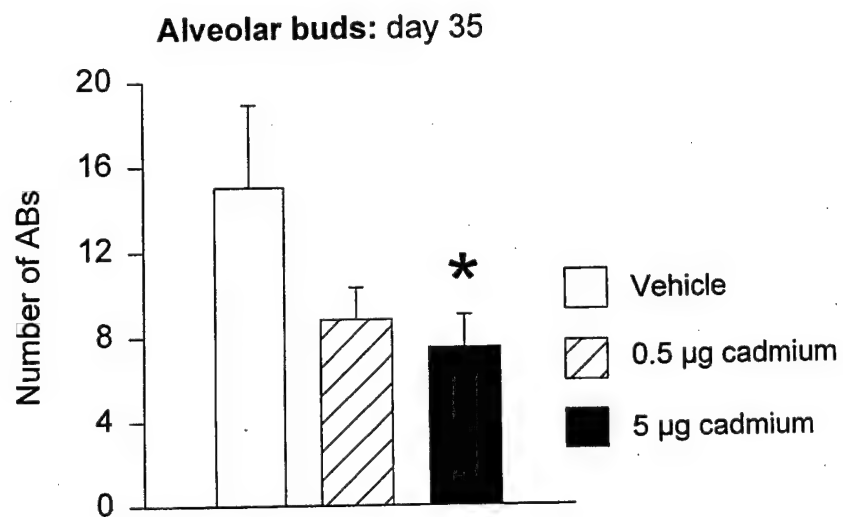
Estrogens, BRCA1, and Breast Cancer[1]

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Abstract

Findings obtained in *in vitro* assays and animal studies indicate that estrogens might influence the activity of the tumor suppressor gene BRCA1, and BRCA1 in turn may suppress the activity of the estrogen receptor. This review will discuss the possibility that interactions between estrogens and BRCA1 partly explain why elevated circulating estrogen levels appear to increase breast cancer risk among postmenopausal women but not among young women. A hypothesis is proposed that estrogens have a dual role in affecting breast cancer risk. In young women whose breasts have not yet accumulated critical mutations required for cancer initiation and promotion, activation of BRCA1 by estrogens helps to maintain genetic stability and induce differentiation, and therefore estrogens do not increase breast cancer risk. Breasts of older women, in contrast, are likely to contain transformed cells whose growth is stimulated by estrogens. Although BRCA1 is also probably activated by estrogens in older women, its function may have been impaired, for example, due to increased methylation associated with aging. Estrogen exposure in women who carry germ-line mutations in BRCA1 may always increase breast cancer risk because estrogens would be able to cause DNA damage and increase genetic instability without being opposed by BRCA1-induced repair activity. This might lead to an increase in the number of overall mutations, including those that initiate breast cancer. In addition to increasing genetic instability, reduced BRCA1 activity may also be linked to changes in the mammary gland morphology that predispose individuals to breast cancer. For example, a persistent presence of lobules type 1, which are the least differentiated lobular structures in the human breast, is seen in the BRCA1 mutation carriers. The aim of this review is to discuss the role of premenopausal estrogens in breast cancer and to initiate more research that would lead to novel means of reducing breast cancer risk, particularly among BRCA1 mutation carriers.

Introduction

The role of estrogens in affecting breast cancer risk during premenopausal years has remained largely unknown. Several factors related to reproduction appear to predispose women to breast cancer. For example, women with early onset of menarche (menstruation begins at <12 years) or late menopause (menopause occurs after 55 years) have an increased risk of developing breast cancer (1). These findings suggest that the longer the exposure to ovarian estrogens, the higher the risk. This view is supported by the fact that surgically induced menopause before age 45 years and the resulting removal of ovarian estrogens markedly reduce breast cancer risk (2–5). Furthermore, the partial ER[3] agonist tamoxifen, which blocks the actions of estrogens in the breast, effectively prevents primary and recurring

breast tumor development (6). However, it is not clear that there is a correlation between high estrogen exposure and high breast cancer risk during the years when women have functional ovaries. In fact, an increase in breast cancer risk may be seen after a modest reduction in circulating estrogens, such as that produced by unilateral ovariectomy (4, 5), oral contraceptive (7, 8) or contraceptive depot use [both of which inhibit ovulation and ovarian estrogen production (9, 10)], or low body weight and low fat intake (11–14). In contrast, an increase in exposure to circulating estrogens during premenopausal years caused by several pregnancies (15), short menstrual cycle length (16, 17), high BMI (18), or a high fat intake (11) may reduce the risk of developing breast cancer. High BMI or fat intake are indirect indicators of increased estrogenicity: a considerable amount of estrogen production occurs in adipose tissue, which is a site for conversion of adrenal androgens to estrogens, particularly in prepubertal girls and postmenopausal women.

A hypothesis is proposed here that estrogens might play a dual role in affecting breast cancer risk. On one hand, there is evidence to indicate that estrogens might serve as preinitiators, initiators, and promoters of breast cancer. We generally associate estrogens with promotion of the growth of existing malignancies in the breast (Fig. 1). However, these hormones and their metabolic products are also shown to induce direct and indirect free radical-mediated DNA damage, genetic instability, and mutations in cells in culture and *in vivo* (19), suggesting a role for estrogens in cancer initiation. Furthermore, estrogens may serve as preinitiators. For example, elevated fetal estrogen levels can permanently alter the morphology of the mammary gland (20) and cause a persistent presence of epithelial structures (TEBs) that are known to be sites of malignant growth (21). Data obtained in animal models and indirect evidence in humans indicate that high *in utero* estrogenicity increases breast cancer risk (20, 22, 23).

In contrast to these adverse effects of estrogens on the breast, in certain circumstances, such as during pregnancy that occurs before age 20 years (1, 15, 24) and during the prepubertal period and childhood (11, 14, 25), estrogens actually reduce breast cancer risk. The reduced risk could be achieved through estrogen-induced activation of certain tumor suppressor genes, including BRCA1 (26–28) and p53 (29) that are critical in DNA damage repair and in maintaining genetic stability, thus reducing the likelihood that breast cancer will be initiated. The interaction between estrogens and tumor suppressors might be important during the early reproductive years, when the breast does not yet contain any malignancies. Once breast cancer initiation has taken place, estrogens might promote the growth of transformed cells, leading to the development of detectable breast cancer. Because estrogens increase BRCA1 expression in human breast cancer cells *in vitro* (27, 28), they are also likely to do so in women whose breast contains malignant cells. However, given that breast cancer initiation has already occurred, the function of one or more tumor suppressors may be impaired at this point (failure in tumor suppressor gene function is believed to contribute to cancer initiation). In women carrying a mutated *BRCA1* gene, estrogens may always induce genetic instability because the mutated BRCA1 is

PRE - INITIATION
HIGH ESTROGENIC ENVIRONMENT *In utero*

MAMMARY GLAND

INCREASED NUMBER OF TARGETS
FOR MALIGNANT TRANSFORMATION/
CHANGES IN TISSUE ARCHITECTURE

CHANGED PATTERN
OF GENE EXPRESSION

INITIATION AND PROMOTION
HIGH ESTROGENIC ENVIRONMENT

BREAST

ESTROGEN &
METABOLITES

DNA DAMAGE,
GENETIC INSTABILITY
& MUTATIONS

ESTROGEN

MALIGNANT
CELLS

BREAST
WITH CANCER

TUMOR

Fig. 1. Estrogens may increase breast cancer risk by acting as a preinitiator (if the exposure occurs during fetal life), an initiator (by inducing direct or indirect free radical-mediated DNA damage), or a proliferator (stimulating the growth of existing malignant cells in postinitiated breast). The proposed mechanisms by which *in utero* estrogen exposure increases breast cancer risk include a persistent presence of mammary epithelial structures that serve as sites of tumor initiation and changes in the pattern of expression of genes that are relevant to breast cancer.

unable to correct genetic alterations. Evidence is presented below to support this hypothesis.

Why Would Estrogen Exposure during Reproductive Years not Increase Breast Cancer Risk?

In addition to the reproductive system and the breast, estrogens are required for the development and function of many other tissues. Estrogens are of critical importance in bone development and the maintenance of bone density (30, 31) and a healthy cardiovascular system (32, 33). Estrogens are also required for neuronal growth and differentiation, and these hormones are linked to cognitive functions (34) and mood (35, 36). Because estrogens possess several essential functions, it would be surprising if a complementary system did not exist in parallel with estrogens to protect tissues like the breast from the adverse effects of estrogens.

Estrogens exhibit both beneficial and harmful effects on the breast. The breast undergoes periods of varying sensitivity to the adverse effects of estrogens (37). These hormones are needed during normal breast development, particularly during puberty and pregnancy (38). For example, although pregnancy markedly increases circulating estrogen levels, pregnancy before age 20 years reduces breast cancer risk (24). In contrast, a similar pregnancy-induced increase in estrogen levels after age 30 years increases breast cancer risk (1). One explanation for the differential effects of estrogens during pregnancy in younger and older women is that in addition to stimulating epithelial growth, pregnancy estrogens participate in eliminating (by differentiation) those epithelial structures that are most vulnerable to malignant transformation (39). However, differentiation to mature ductal structures is protective only in a breast that does not yet contain any malignant cells, and older women are more likely than young women to have acquired transformed cells, which, when stimulated with estrogens, could lead to breast cancer.

The fact remains that estrogens increase proliferation and genetic instability, perhaps by inducing free radical-mediated DNA damage and mutations (19). Genetic instability, in turn, increases the probability that normal cells are turned to the malignant pathway. It is therefore critical to ensure that whereas estrogens are needed for the optimal function of several important systems, another mechanism(s)

exists that activates DNA repair pathways to respond to the genomic damage initiated by estrogens and that may also occur during rapidly proliferating states. In addition, these genes that are activated by estrogens may also directly regulate the ER, perhaps by suppressing its activity. We propose that a network of normal tumor suppressor genes is activated by estrogens, which then protect the breast from an estrogen-induced increase in genetic damage and, ultimately, from neoplastic changes. Candidate estrogen-regulated tumor suppressor genes are BRCA1 and p53.

Breast Cancer Susceptibility Gene *BRCA1*

Breast cancer is heritable in certain families. In some of these families, a breast cancer susceptibility locus on human chromosome 17q has been identified (40, 41). This locus codes for a tumor suppressor protein BRCA1. Mutations in BRCA1 account for at least 50% of inherited breast cancers and approximately 5% of all breast cancer cases (40).

A murine equivalent to human Brca1, Brca1, is expressed in a wide variety of tissues, including the breast (26). The level of expression is highest in tissues containing rapidly proliferating cells that are also involved in differentiation. In mice, Brca1 is expressed broadly during embryogenesis, whereas after birth, the expression shifts to a more tissue-specific pattern (26). High levels of Brca1 mRNA can be detected in the mouse mammary gland around puberty and during pregnancy. During lactation, Brca1 expression in the mammary gland is low, but it is elevated again after lactation, resulting in higher Brca1 expression in parous mice than in nulliparous mice (26).

The expression pattern of Brca1 during normal development suggests that this gene is tightly linked to the regulation of cellular proliferation (42). This conclusion is supported by observations that BRCA1 mRNA levels exhibit a cell cycle-dependent pattern: expression is low in cells arrested in G_0 or early G_1 and highest at the G_1-S-phase transition (42–44). BRCA1 protein also undergoes hyperphosphorylation during late G_1 and S phases, indicating that the protein is then being activated. However, BRCA1 expression is not limited to cell proliferation. Rajan *et al.* (42) have shown that Brca1 mRNA levels are high in postconfluent HC11 mammary epithelial cells during differentiation and when treated with insulin and glucocorticoids. Because proliferation rates under these conditions are low, and differentiation is high, Brca1 also appears to be involved in the process of differentiation of the breast.

The work by Gowen *et al.* (45) suggests that BRCA1 plays a key role in repairing oxidative DNA damage. This probably indicates that in rapidly proliferating tissues, BRCA1 may help to maintain the integrity of the genetic material. Furthermore, BRCA1 interacts with RAD51, a protein that has been implicated in DNA recombination and repair (46). The fact that BRCA1 has also been identified as a p53-interacting protein (47) lends further support to the idea that BRCA1 may be involved in repairing DNA damage. BRCA1 has been shown to act as a transcriptional coactivator and increase the p53-dependent transcription from P21 and BAX promoters (47). DNA-damaging agents trigger a transient induction of p53, and this gene has been strongly implicated in DNA damage repair.

Besides p53, several other proteins that interact with BRCA1 have been identified, including c-myc, BAP-1 (48), and retinoblastoma susceptibility gene RB1 (49). The c-myc oncogene is closely linked to breast carcinogenesis, and it is one of the early response genes activated in G_1 phase, resulting in the activation of a number of other genes with important roles in cell cycling. It has been suggested that BRCA1 down-regulates c-myc activity (48). BAP-1 is a novel protein found on the basis of its interaction with BRCA1 (50). BAP-1 might enhance BRCA1-mediated growth inhibition, at least in human breast

cancer cells (50). Inherited mutations in one of the RB1 alleles result in the development of retinoblastoma and/or osteosarcoma and increase susceptibility to other cancers. It was recently shown that the product of the *RB1* gene, Rb, regulates the expression of both the murine *Brca1* and human *BRCA1* genes (49). BRCA1 also transactivates the expression of p21, the major cyclin-dependent kinase inhibitor involved in the inhibition of cell cycle progression and induction of apoptosis, in a p53-independent manner (51).

In summary, BRCA1 has been implicated to have a primary role in DNA damage response by processing signals that arise after damage (48). This role results from cross-talks with other critical elements of signal transduction pathways and causes cell cycle arrest, DNA repair, and perhaps apoptosis.

Estrogens and BRCA1

The fact that Brca1 expression is induced during puberty and pregnancy, when estrogen levels are dramatically increased, suggests that estrogens might stimulate the expression of this gene. This suggestion is supported by a finding showing that E_2, together with progesterone, increases the level of Brca1 expression in the mammary glands of ovariectomized mice (26). Studies in ER-positive MCF-7 and BT20T human breast cancer cells indicate that depletion of estrogens significantly reduces BRCA1 mRNA expression, and the expression is increased again by treatment with E_2 (27, 28). It should be noted that no estrogen-responsive element has been identified within the promoter of the *BRCA1* gene, and the increase in BRCA1 mRNA expression by estrogens probably occurs via an estrogen-initiated increase in overall RNA synthesis (52).

It is essential to determine how and why estrogens stimulate BRCA1 expression. BRCA1 mRNA expression and ER mRNA expression are closely linked to each other, suggesting a functional relationship between the two genes (53). Furthermore, methylation of the BRCA1 promoter appears to be strongly correlated with a lack of ER or progesterone receptor expression (54). In accordance with these observations, BRCA1 was recently shown to have an ability to regulate the cellular response to estrogens (55). In *in vitro* studies conducted using human breast cancer cells, BRCA1 protein inhibited ER-α-mediated transcriptional pathways related to cell proliferation. This finding suggests that in addition to maintaining genomic stability during periods of rapid cellular division and multiplication, BRCA1 may also suppress signaling initiated by estrogen-induced activation of ER-α. Thus, during puberty and pregnancy, when estrogens and BRCA1 expression are both significantly increased, the function of BRCA1 may be to protect the breast from estrogen-induced genetic instability by inhibiting ER-mediated pathways, inducing differentiation, and repairing genetic damage. BRCA1 might also be particularly important in controlling cellular proliferation. A loss of BRCA1 function leads to increased proliferation of malignant cells in cell culture (56, 57), and stable transfection of wild-type BRCA1 into these cells inhibits their growth (58). However, activation of BRCA1 seen during puberty and pregnancy does not seem to block proliferation occurring in the breast at these times.

BRCA1 and Breast Cancer

Germ-line mutations only occur in one BRCA1 allele because homozygous deletion of BRCA1 is lethal *in utero*. However, germ-line BRCA1 mutation carriers who develop breast cancer often exhibit loss of heterozygosity of the wild-type BRCA1 locus (59). Thus, both BRCA1 alleles appear to be lost in those breast cancer cases where BRCA1 is the precipitating genetic lesion. It is possible that a loss of one allele, through a germ-line mutation, may alter the function of other genes, leading to a dramatic genomic instability. This instability then creates an environment in which the loss of function of wild-type BRCA1 is highly likely. It is not inconceivable that loss of wild-type BRCA1 is not a causative factor in the development of inherited breast cancer but rather a side effect.

Somewhat surprisingly, somatic mutations in BRCA1 are extremely rare in sporadic breast cancer (40, 60). Instead, in sporadic breast cancers, the level of normal BRCA1 protein is often reduced either through loss of heterozygosity of one BRCA1 allele or by other means (56, 61). Down-regulation of the normal BRCA1 may be caused, for example, by alternative splicing (62), aberrant methylation (63–65), or defects in subcellular localization of the BRCA1 protein (66). Failure of transcriptional regulation by ER may also be responsible for reduced BRCA1 mRNA levels in sporadic breast cancer (67).

The level of BRCA1 expression in sporadic breast cancer is related to the degree of invasiveness of the tumor. Compared with normal breast tissue, BRCA1 expression is lowest in invasive cancer (56) and is intermediately reduced in *in situ* carcinomas (68). However, the latter finding has not been confirmed in all studies, and there are reports indicating higher BRCA1 mRNA levels in ductal carcinoma *in situ* than in normal mammary epithelium (56). The decreased BRCA1 expression might be a causal event, reflecting tumor progression, or a secondary effect caused by changes in upstream regulatory pathways controlling BRCA1 expression (69). In either case, reduced BRCA1 expression rather than loss of function of both alleles is linked to sporadic breast cancer.

Taken together, BRCA1 mutations appear to cause breast cancer only when present in the germ line, although the reasons for this are unknown. Furthermore, in germ-line mutation carriers, both alleles are lost at the time the tumor is detected, whereas in sporadic breast cancer, BRCA1 expression is reduced but not completely lost. This suggests that a loss of function of wild-type BRCA1 in germ-line mutation carriers has to occur, whereas this is not the case in sporadic breast cancer. It is possible that BRCA1 may function differently in embryonic *versus* adult cells (69). During embryogenesis, BRCA1 is of critical importance for cell proliferation; thus, homozygous germ-line lesions result in an early cell proliferation defect that kills the embryos. Consequently, cellular events leading to breast cancer might be different in BRCA1 mutation carriers *versus* women who develop sporadic breast cancer but exhibit reduced BRCA1 expression as adults. In the mutation carriers, the presence of only one functional BRCA1 allele *in utero* may create an environment of increased genetic instability, increasing the probability that mutations in other critical genes will occur. This argument is supported by the fact that p53, another tumor suppressor gene, which is called "the guardian of the genome," is more frequently inactivated in BRCA1 mutation-associated tumors than in sporadic breast cancer (70, 71). Up to 90% of BRCA1 mutation-associated tumors harbor a p53 mutation and/or p53 protein accumulation [which occurs due to either a mutation in the *p53* gene or alterations in p53 upstream signaling pathways (71)]. These events might also lead to a loss of wild-type BRCA1, which may or may not be essential for tumor initiation.

In sporadic breast cancer, normal BRCA1 function might be reduced due to various environmental factors. The environmental exposures that may alter BRCA1 expression levels in women who develop sporadic breast cancer include PAHs or changes in circulating estrogen levels. PAHs are widely present in our environment, and they reduce BRCA1 mRNA expression in human breast cancer cells (72). We propose that BRCA1 levels might be reduced by an exposure to a low estrogenic environment. Changes in estrogen exposure levels can be caused by differences in the amount of adipose tissue or differences in the use of contraceptive drugs, hormone replacement therapy, or exposure to environmental estrogens. It is possible that an

interindividual variability in estrogen levels in women contributes to the lowering and increasing of BRCA1 expression.

There is some indirect evidence that estrogen-induced activation of BRCA1 may be important in protecting the breast. For example, the phytoestrogen genistein (73), which is a major active component in soy products and may reduce the risk of developing premenopausal breast cancer (74), increases the expression of BRCA1 in human breast cancer cells in culture (75). Because genistein exhibits weak estrogenic activities, and estrogens also up-regulate BRCA1, the results suggest that estrogenic compounds may reduce breast cancer risk by activating normal BRCA1. Another observation in support of the estrogen/Brca1 link is that high estrogenicity before the onset of puberty in animal models reduces breast cancer (76–78), and Brca1 is up-regulated at puberty (26). Increased estrogenicity before the initiation of ovarian estrogen production might activate BRCA1 earlier than normal, which could further help to maintain genomic stability at puberty. The surge of ovarian estrogens at puberty is likely to increase estrogen-induced DNA damage and impair repair mechanisms. This speculation is based primarily on results obtained in animal models. However, high prepubertal estrogenicity might also protect the human breast. It has been noted that in humans, indicators of high estrogenicity during childhood and early premenopausal years are linked to inhibition rather than initiation of breast cancer (14, 25). Thus, estrogenic exposures at these times, perhaps through estrogen-induced activation of BRCA1, may be involved in reducing the probability that normal cells would later turn to the malignant pathway.

It has been suggested that the rarity of BRCA1 mutations in sporadic breast cancer is due to the greater likelihood of BRCA1 inactivation by nonmutational mechanisms than by mutation. One nonmutational mechanism of BRCA1 inactivation that has been observed in sporadic breast cancer is methylation (64). Hypermethylation of CpG-rich areas located within the promoter of genes may be a common mechanism of silencing tumor suppressor genes. Hypermethylation has been shown to increase with age (79), and if this occurs in the *BRCA1* gene, it could help to explain why estrogens increase breast cancer risk in older women. Theoretically, exposing older women to estrogens results in tumor promotion that methylated BRCA1 cannot prevent. Besides aging, it is not known what induces hypermethylation in women with sporadic breast cancer. One of the pathways could be through an exposure to various environmental agents that promote hypermethylation of important cancer-related genes (80), possibly including BRCA1. The possibility also exists that low circulating E_2 levels might contribute to the induction of hypermethylation (81).

BRCA1 Mutation Carriers and Estrogens

If one of the BRCA1 alleles is lost due to a mutation, as is the case in familial breast cancer, estrogens might be more likely to cause genomic instability than if both alleles were functioning normally. This would mean that estrogen exposure, particularly during puberty and young adulthood, increases the penetrance of breast cancer in germ-line BRCA1 mutation carriers. Although approximately 70% of women who carry a germ-line BRCA1 mutation will develop breast cancer by age 70 years (82), the remaining 30% do not. It is not known whether the age at onset of puberty or menopause, circulating estrogen levels, body weight, diet, exercise, alcohol intake, or other factors that affect estrogen levels alter breast cancer risk among germ-line BRCA1 mutation carriers. Oral contraceptives, when used before first pregnancy, may increase breast cancer risk in BRCA1 carriers (83). In contrast, smoking appears to reduce breast cancer risk in these women (84). Oral contraceptive use reduces the exposure to ovarian estrogens, and exposure to the synthetic estrogen is lower than

that to estrogen originating from the ovaries. Smokers are reported to have lower circulating estrogen levels than nonsmokers, although the association has not been confirmed in all studies (85–87). Thus, based on these observations, it cannot be determined whether high levels of estrogens increase breast cancer risk in women with BRCA1 mutations.

However, there are four important observations that suggest that estrogens may indeed increase the penetrance of breast cancer in BRCA1 mutation carriers. First, men heterozygous for BRCA1 mutations do not exhibit an increased incidence of breast cancers (88), indicating that low estrogen and/or high androgen levels might be protective. Second, bilateral prophylactic ovariectomy is associated with a significantly reduced breast cancer risk in women who carry a BRCA1 mutation (89). Third, women possessing germ-line mutations in BRCA1 are particularly susceptible to breast cancer as a result of pregnancy (90, 91). Pregnancy increases circulating estrogen levels by approximately 10-fold. Fourth, women with a strong family history of breast cancer (approximately 50% of these women are BRCA1 mutation carriers, and most of the others carry a mutation in some other tumor suppressor gene) exhibit a 4-fold increase in breast cancer risk if they had a high BMI at the age of 12 years (92). As indicated above, a high BMI during childhood clearly reduces sporadic breast cancer risk (14, 25). The last two findings strongly suggest that a mutated BRCA1 cannot protect the breast from the cancer-initiating/promoting effects of estrogens.

Women who do not carry germ-line BRCA1 mutations but have lost the function of the normal *BRCA1* gene by other means should also exhibit an estrogen-induced increase in breast cancer risk. Such a loss may be more likely to have occurred in older *versus* younger women. Relatively consistent evidence shows that elevated estrogen levels during postmenopausal years increase breast cancer risk. Postmenopausal women who have high circulating estrogen levels (93, 94), are obese (95), or are exposed to hormone replacement therapy (96) exhibit an increase in breast cancer risk, although not all studies support these findings. The probability of genetic mutations is believed to increase with age, but no evidence exists thus far to indicate that the *BRCA1* gene is mutated in older women (or in young women, for that matter) who develop sporadic breast cancer. However, older women may have acquired mutations in genes in which BRCA1 acts as a coactivator, such as p53, and this could potentially lead to a reduction in BRCA1 activity as well. As discussed above in connection to the differential effect of pregnancy on breast cancer risk in young and older women, breasts of older woman are more likely to contain preneoplastic and neoplastic cells than those of young women. Based on what we currently know about cancer initiation (loss of function of tumor suppressor genes and overexpression of oncogenes allow normal cells to turn to a malignant pathway), the function of one or more tumor suppressor genes, possibly including BRCA1, in women with preneoplastic lesions is more likely to have been lost in older women than in young women. Methylation of BRCA1 is also among the potential mechanisms that could inactivate this gene in older women (64, 79). Thus, although estrogens stimulate BRCA1 in older women, BRCA1 is not able to repair and maintain genomic stability because it has lost its function (or function is impaired) in the process that has allowed preneoplastic lesions to occur in the first place.

Estrogens, BRCA1, and Mammary Gland Morphology

It is generally believed that mutations in tumor suppressor genes and oncogenes are required for breast cancer initiation to occur. However, alterations in normal communications between stroma and parenchyma, perhaps reflecting or occurring in parallel with epige-

netic changes, might be essential in tumor formation. It has been argued that the structure of the breast tissue has to be critically altered for malignant transformation to progress, even in the presence of multiple chromosomal mutations (97). We have been studying changes in the mammary epithelial tree in mice and rats exposed to estrogenic compounds during the *in utero* period. *In utero* estrogenic exposures that increase breast cancer risk in animal models increase the number of TEBs in the offspring's mammary gland and prevent their differentiation (20, 98). TEBs contain a rapidly proliferating population of epithelial cells and drive ductal growth (21). In mice and rats, the presence of TEBs is highest around puberty, and the TEBs subsequently differentiate to lobulo-alveolar units, becoming virtually nonexistent in the adult gland (21). Animal data indicate that TEBs are the primary targets for carcinogen-induced malignant growth (21), and the corresponding structure in the human breast, terminal ductal lobule unit, may also be the site most susceptible to the development of human breast cancer (99). Interestingly, in animals, the mammary structure exhibiting the highest level of Brca1 mRNA is the TEB in virgin animals and the alveoli during pregnancy (26).

It was noted recently that breasts of women who are germ-line BRCA1 mutation carriers exhibit a high number of the least differentiated lobules type 1, regardless of whether they are parous or not (100). Normal (noncarrier) women show a long-lasting reduction in the number of lobules type 1 and an increase in the number of well-differentiated lobules type 3 after pregnancy (101). It is possible that the persistent presence of lobules type 1 in the BRCA1 mutation carriers results from an interaction between high *in utero* estrogen exposure and one nonfunctional BRCA1. Another possibility is that one mutated *BRCA1* gene is sufficient to prevent normal differentiation of the human breast that occurs during pregnancy.

High estrogenicity during fetal life may contribute to high breast cancer incidence among BRCA1 mutation carriers. Although estrogen levels are significantly higher in pregnant women than in nonpregnant women (102), there is still a 4–6-fold variability in these levels among women who are undergoing apparently normal pregnancies. Thus, some pregnant women (and their fetus) are exposed to significantly higher levels of estrogens during pregnancy than other pregnant women. It has been hypothesized that the highest range of fetal estrogen exposure levels increases later breast cancer risk compared with the lowest range of fetal estrogen exposure levels (22, 103). This hypothesis is supported by indirect epidemiological evidence showing that high birth weight, which is linked to a high fetal estrogenic environment (104), increases breast cancer risk (105, 106). Dizygotic twins also are exposed to an increased fetal estrogenic environment and exhibit increased breast cancer risk as adults (107, 108). However, a recent study comparing pregnancy estrogen levels between Asian and Caucasian women suggests that high pregnancy E_2 levels may not increase breast cancer risk if birth weight is not simultaneously increased (109).

In accordance with the changes seen in the mammary gland morphology in animals exposed to high *in utero* estrogenicity, high placental weight in humans, which indicates high fetal estrogen exposure, is associated with high density mammographic parenchymal patterns (110). High density mammographic patterns, in turn, are associated with increased breast cancer risk (111). It remains to be determined whether or not the high number of lobules type 1 in the breasts of BRCA1 mutation carriers reflects high fetal estrogen levels, and whether or not they contribute to increasing breast cancer risk in these women also remains to be determined.

Another observation suggesting a link between high *in utero* estrogenicity and breast cancer in BRCA1 mutation carriers is that *in utero* exposure to a high estrogenic environment reduces total ER content (including both the classical ER-α and novel ER-β subtypes) in the

normal mammary gland and in breast tumors (112–114). Breast cancers in BRCA1 carriers are often ER-α negative (115, 116). This could indicate that germ-line BRCA1 mutation carriers who develop breast cancer are those who also were exposed to the highest range of estrogen levels during fetal life, which then down-regulated ER expression in the breast. However, it can also be argued that continuous adult exposure to high estrogen levels both down-regulates breast ER levels and increases breast cancer risk in women who are BRCA1 mutation carriers. Whether or not low ER levels in the breast are causally related to the development of breast cancer in the mutation carriers is not known.

Brca1 and Mammary Tumorigenesis in Animal Models

In animal models, loss of one Brca1 allele is not sufficient to promote cancer. For example, although homozygous deletions of Brca1 in knockout mouse models are lethal early in embryonic development (117–119), mice carrying heterozygous deletions of Brca1 are phenotypically normal and do not exhibit an increased predisposition to tumorigenesis (120). However, when the remaining *Brca1* gene is inactivated in mature heterozygous brca1 knockout mice, mice will develop breast cancer (121).

There is no evidence that reduced *Brca1* expression is related to carcinogen-induced rodent breast cancer models or to models in which mammary tumors are seen in mice exhibiting activated *neu* or activated *ras* oncogenes. This appears to contradict the human data showing reduced BRCA1 expression in sporadic breast cancer *versus* normal tissue. Brca1 mRNA expression levels are similar in mammary tumors induced by carcinogens 7,12-dimethylbenz(a)antracene or methylnitrosourea or by activation of *neu* or *ras* oncogenes and in nonmalignant cells (122). The key to understanding the apparent species difference in BRCA1 expression in tumors and nonmalignant tissues might lie in the factors that cause breast cancer in women *versus* rats. In women, the underlying genetic and molecular events that initiate breast cancer have remained largely unknown, whereas in animal models, the causal factor is apparent (for example, carcinogen exposure or overexpression/knockout of a specific gene). Environmental exposures and changes in hormonal status might play a major role in human breast cancer, although the details are far from being clear. It is possible that these hormonal/environmental factors lead to reduced BRCA1 expression. For example, a recent study indicates that the PAH benzo(a)pyrene reduces BRCA1 mRNA levels in MCF-7 human breast cancer cells (72). This, in turn, might cause increased genetic instability and cell proliferation, mutations in a gatekeeper gene, and, finally, breast cancer. In most rodent models, it is carcinogens and oncogenes that induce mammary tumors, not hormonal/environmental factors, although they clearly affect the promotion and progression stage of rodent mammary tumorigenesis and may serve as preinitiators (20).

Tumor Suppressor Gene p53

Germ-line and somatic mutations in the p53 tumor suppressor gene predispose carriers to a wide variety of cancers, including breast cancer (123). Mutation of p53 is the most common somatic alteration in sporadic breast cancer, with an estimated frequency of 12–46% in invasive breast cancers (124). p53 has an ability to recognize and bind to damaged DNA, repair it, and induce both cell cycle arrest and apoptosis (125). p53 has thus been categorized as both a caretaker and gatekeeper tumor suppressor gene (126).

Like BRCA1, expression of p53 mRNA may be modulated by estrogens. T47D human breast cancer cells exhibit a reduction in p53 expression when grown in medium depleted of endogenous steroids, and subsequent E_2 administration increases p53 expression (29). Fur-

A NORMAL MAMMARY GLAND
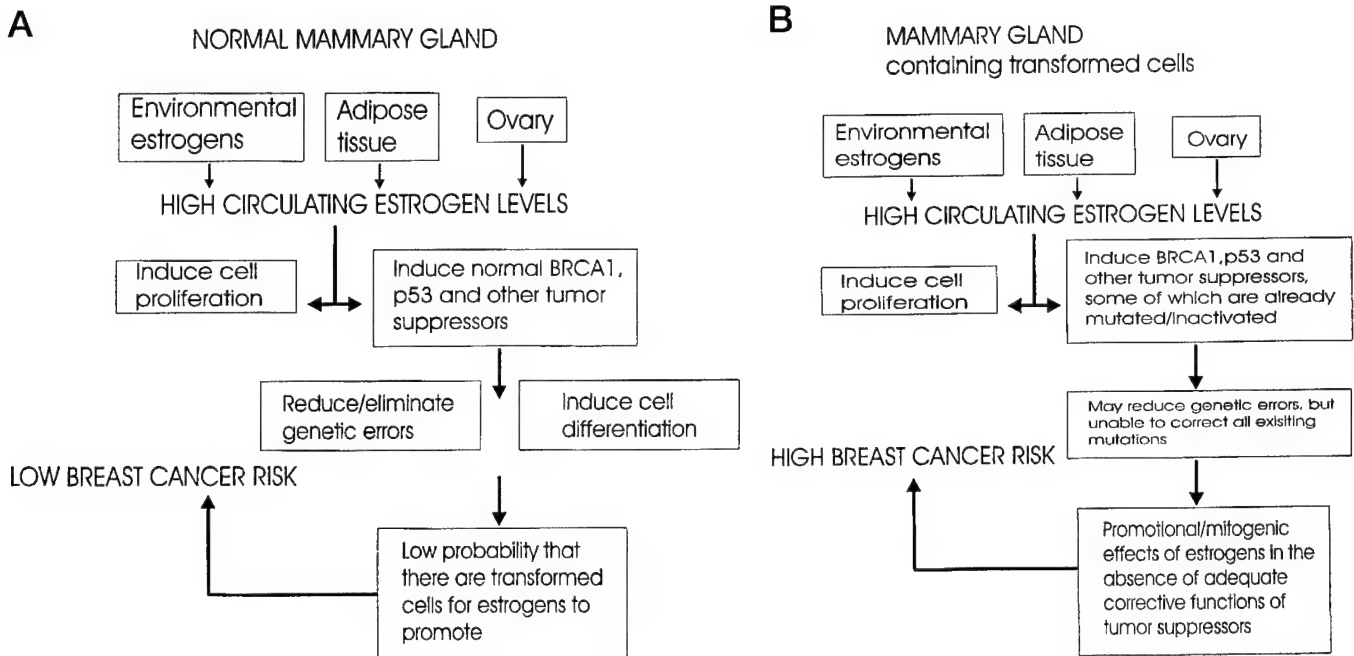

B MAMMARY GLAND
containing transformed cells

Fig. 2. Proposed effects of high circulating estrogen levels (originating from the environment, adipose tissue, and ovaries) on breast cancer risk in (A) normal breast (for example, in young women) and (B) breast containing transformed cells (for example, in older women or in women with germ-line BRCA1 mutations). In normal breast, estrogens stimulate normal tumor suppressor genes, which then reduce and eliminate genetic errors and may induce cell differentiation, leading to reduced breast cancer risk. In the breast containing transformed cells, estrogens also stimulate tumor suppressor genes, but because at least some of them are already mutated/inactivated, they are not able to correct existing mutations or induce differentiation. In this breast, estrogens further promote the growth of transformed cells, leading to the development of detectable breast cancer.

thermore, E_2 administration increases p53 expression in human endometrial adenocarcinomas growing in nude mice (127). In hamster kidney, chronic administration of the synthetic estrogen diethylstilbestrol also increases p53 expression (128). In addition to the fact that both p53 and BRCA1 seem to be induced by estrogens and play a role in DNA repair, their relationship is shown to be more than merely general. Several lines of evidence indicate that p53 is associated with breast cancer in BRCA1 mutation carriers. First, mutations in p53 occur at a high frequency in tumors of BRCA1 mutation carriers (70). Second, BRCA1 enhances p53-mediated transcription (51), as evident from the observation that transfection of cells with mutated BRCA1 inhibits p53-mediated transcription of effector genes (47). Furthermore, BRCA1 stimulates many p53-responsive genes, although it can also stimulate expression of these genes independent of p53 (47). A mutation in p53 may also be associated with down-regulation of BRCA1 in women who develop sporadic breast cancer.

It has been suggested that because E_2 promotes human breast cancer cell proliferation, the induction of p53 may indicate that *in vitro* E_2 stimulates p53 to regulate proliferation (129). We propose that this also applies *in vivo* in the human breast. Thus, premenopausal women exposed to elevated estrogen levels may also exhibit an increase in p53 expression. If p53 is normal, it guards the genome against somatic mutations that might initiate cancer; if it is mutated or silenced by other means, it is unable to prevent increased genetic instability induced by estrogens, and breast cancer risk is increased.

Other Tumor Suppressor Genes

Besides BRCA1 and p53, other tumor suppressor genes have been identified, including BRCA2. In theory, they may protect the breast from the adverse effects of estrogens. For example, the *BRCA2* gene on chromosome 13q is another tumor suppressor gene linked to heritable breast cancer (130, 131). There are striking similarities in the expression patterns and functions of Brca1 and Brca2 (26, 69). Brca2 is expressed in the same tissues and cell types as Brca1 (42, 132).

Furthermore, BRCA2 expression is also induced by estrogens (28) and is high during puberty and pregnancy (42, 132). It is plausible that many other tumor suppressor genes will be identified in the breast that are either stimulated or inhibited by estrogens or independent of these hormones. Estrogens at different time points during development, as well as the level of estrogenicity originating from ovaries *versus* that of non-gonadal estrogens, are likely to affect breast cancer risk in a manner that is determined by a response to the total network of signaling pathways of estrogens.

Conclusions

There is a considerable amount of confusion among scientists and lay people as to whether the risk of breast cancer can be reduced by altering lifestyle, including dietary modifications and exercise patterns. A low-fat diet is known to reduce serum estrogen levels, but low body weight does not reduce premenopausal breast cancer risk (11–14, 25). A high fiber content reduces circulating estrogen levels by increasing fecal excretion of the hormone (133) but does not consistently reduce breast cancer risk (134). Similarly, exercise reduces estrogen levels (135) without necessarily reducing the risk of developing breast cancer (136). The consistent findings indicating that an early onset of puberty increases breast cancer risk are believed to support the idea that early estrogen exposure increases breast cancer risk; however, it may merely reflect an exposure to an elevated *in utero* estrogenic environment because this environment both accelerates puberty onset and increases breast cancer risk (20). Therefore, it is of critical importance to clarify the link between estrogens, particularly changes in estrogenicity induced by lifestyle factors, and human breast cancer.

We propose that estrogens have a dual role in affecting breast cancer risk by interacting with tumor suppressor genes on one hand and by stimulating cell proliferation, as summarized in Fig. 2, on the other hand. Kinzler and Vogelstein (126) describe BRCA1 as a "caretaker" tumor suppressor gene. A caretaker's role is to maintain

the integrity of the genome. Thus, high estrogen levels may increase normal BRCA1 expression in an attempt to ensure genomic stability in the face of a potential estrogen-induced increase in genomic damage. Mutated BRCA1 in inherited breast cancer or down-regulated BRCA1 in sporadic breast cancer is unable to repair genomic damage induced by high levels of estrogens, increasing the likelihood that other mutations will occur and that a normal cell will ultimately become transformed. This interaction between estrogens and BRCA1 probably explains, at least in part, why BRCA1 mutation carriers exhibit a significantly increased risk of breast cancer and a moderately increased cancer risk in other estrogen-regulated sites (ovaries, prostate, and possibly the colon), but not in non-estrogen-regulated tissues. One way to test the hypothesis that an interaction between estrogens and BRCA1 determines whether estrogens increase or reduce breast cancer risk is to determine BRCA1 expression levels in relation to BMI, fat intake, or circulating estrogen levels in women to find out whether these factors are associated with tumor suppressor activity. Another way to test the hypothesis is to determine whether women who carry a germ-line BRCA1 mutation show an increase in breast cancer penetrance when exposed to the highest levels of estrogens *in utero* (*i.e.*, women who had a high birth weight) or during puberty (*i.e.*, women who consumed a high-fat diet or had a high BMI). If this turns out to be true, perhaps penetrance of breast cancer in BRCA1 mutation carriers can be reduced by dietary modifications that reduce pregnancy estrogen levels and body weight throughout life.

Acknowledgments

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Genistein: Does It Prevent or Promote Breast Cancer?

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Diet is estimated to contribute to approximately 50% of all newly diagnosed breast cancers. As such, a search for dietary factors differentially consumed among populations with increased breast cancer risk (e.g., Caucasians) compared to those with low risk (e.g., Asians) has become a priority. One such dietary component, which is typical to the Asian but not the Caucasian diet, is soy. We review data relevant to attempts to determine whether soy, and more specifically genistein, is a dietary component that may help to explain the dramatic disparity in breast cancer risk among these populations. *Key words:* antiproliferative effects, breast cancer, estrogenic effects, genistein. *Environ Health Perspect* 108:701–708 (2000). [Online 23 June 2000]
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Epidemiologic data indicate a great disparity between breast cancer risks in Western and Eastern countries. Historically, the risk of American women developing breast cancer has been as high as 7 times that of Asian women (*1*). Today the disparity in risk is similarly significant, although the difference in incidence between Western and Eastern countries has narrowed slightly. For example, one in eight white women in the United States can expect to develop breast cancer in her lifetime; this risk is roughly 5-fold less in Japanese and Chinese women residing in Asia (*2*). However, extensive migration studies indicate that Asian women who immigrate to the United States and adopt a Western lifestyle develop risk comparable to Caucasian women within two generations (*3,4*). These studies provide strong evidence in support of other epidemiologic studies showing that 5–10% of breast cancer cases are estimated to be attributable to inherited factors, and thus > 90% of newly diagnosed breast cancers may be caused by unspecified factors probably related to lifestyle. Ziegler et al. (*5*) investigated the link between age of immigration to the United States and increased breast cancer risk in Chinese, Japanese, and Filipino women. The authors found a strong correlation between early age of immigration (< 35 years of age) and a marked increase in breast cancer risk (*5*). In fact, Asian women born in America, compared to their counterparts born in the East, had a 60% higher risk of breast cancer. Additionally, in all three ethnic groups, immigrants living in the United States for more than a decade had a significantly greater risk than more recent immigrants (*5*).

It is clear from both epidemiologic and clinical data that exposure to estrogens has significant influences on breast cancer development. Estrogens induce the proliferation of normal and malignant mammary cells, and are thus linked to breast cancer promotion and progression. Interestingly, a number

of reports indicate that Asian women living in Asia have up to roughly 40% lower serum estrogen levels than Caucasian women living in the United States or Britain (*6,7*). Based on these data, it is increasingly clear that the protective effect seen in Asian countries does not correlate with genetic influences, but rather, with environmental and lifestyle factors. Thus, it has long been the goal of innumerable scientists to isolate those factors that may be responsible for the dramatic disparity in breast cancer risk between Caucasian and Asian women.

Diet is estimated to contribute to up to 50% of all newly diagnosed breast cancer cases (*8,9*). One particular class of dietary compounds that has received much attention, based on their high concentration in potentially protective foods and their reported antiproliferative effects, is phytoestrogens. Consumption of phytoestrogens, particularly soy products, as well as legumes, is higher in Asia than in the Western world (*10*). Soy-based diets are high in genistein (4,5,7-trihydroxyisoflavone), which has been widely studied for its potential anticancer properties. The exact mechanism by which genistein may exert its antitumorigenic effects is not clearly understood; however, it is a specific and potent inhibitor of both protein tyrosine kinases and topoisomerase II (*11,12*). Furthermore, genistein is able to inhibit angiogenesis and metastasis in some tumor models and to selectively reverse multidrug resistance protein-associated multidrug resistance in *in vitro* studies (*13–15*). Recently, genistein's ability to inhibit the cytochrome P450 enzyme CYP1A1 has been described (*16*). The inhibition of CYP1A1 may lead to a reduction in the production of DNA-damaging carcinogen metabolites and may be one mechanism by which genistein can protect against carcinogenesis (*16*). Given reports of its antiproliferative abilities, genistein appears to be a potentially powerful weapon in the breast cancer prevention

and treatment arsenal. A recent review by Barnes (*17*) discussed the possible protective role of genistein in breast cancer, However, at closer look genistein may not be all it is touted to be.

Estrogenic Effects of Genistein

The phytoestrogen genistein is present naturally as several β-glucosides, which are metabolized by intestinal microflora to genistein (*15*). Genistein, a planar molecule with an aromatic A ring, has a chemical structure similar to steroidal estrogens, and its ability to behave as an estrogen in various tissues has been widely described. Observations of phytoestrogens' estrogenic properties date back to the 1950s, when it was discovered that the diadezan metabolite equol was the compound responsible for reduced reproductive capacity in sheep grazing on clover. Subsequently, countless studies have been conducted to characterize the hormonal effects of phytoestrogens including genistein's estrogenic and presumed antiestrogenic properties.

Genistein has significant estrogenic properties in both *in vitro* and *in vivo* models (Table 1). Genistein binds to the estrogen receptor (ER), although its binding affinity is several-fold weaker than that of estradiol (*30*). Genistein can also activate a number of estrogen-responsive genes *in vitro*, including *pS2* and c-*fos* (*18,31*). Furthermore, when administered at low doses, genistein stimulates the growth of ER-positive (ER+) breast cancer cells (*18–20*). Findings in other tissue systems support the estrogenicity of genistein. For example, genistein is uterotrophic in a variety of species, resulting in impaired reproductive activity and increases in uterine wet weights (*21,25,26*). It is important to note that some studies have failed to see any effect of genistein on the uterus, including alterations in

Table 1. Estrogenic effects of genistein.

Observation	Reference
Inhibition of CYP1A1	(16)
Stimulation of ER+ human breast cancer cells *in vitro*	(18–20)
Stimulation of human ER+ breast cancer cells *in vivo*	(18)
Stimulation of rodent mammary gland	(18,21,22)
Stimulation of human breast	(23,24)
Stimulation of reproductive tissues	(21,25,26)
Estrogenic effects on bone, cardiovascular system, and lipid profiles	(27–29)

wet weight (32,33). Furthermore, findings with coumestrol, a more estrogenic phytoestrogen than genistein, indicate that although coumestrol increases uterine wet weights, it does not increase uterine DNA content or alter other indicators of more true estrogenic activity (34). Thus, an increase in uterine wet weight alone does not necessarily indicate that genistein has estrogenic properties.

In addition to directly binding to the ER, genistein may indirectly affect estrogenicity through inhibition of the cytochrome P450 enzyme CYP1A1. It has recently been shown that genistein is a noncompetitive inhibitor of the CYP1A1 enzyme, which apart from playing a role in the metabolism of carcinogens, is responsible for the metabolic degradation of 17β-estradiol. Thus, it is possible that genistein-mediated inhibition of estradiol degradation could result in higher levels of circulating estradiol and thus elevated ER activity (16).

Genistein has estrogenic effects on the hypothalamic/pituitary axis in ovariectomized Sprague-Dawley rats (21). Human studies indicate that high soy intake can disrupt the hypothalamic/pituitary/gonadal axis in premenopausal women similar to that seen in animal models (23,35). This perturbation is not seen in postmenopausal women (36), suggesting a differential effect of soy/genistein on pre- and postmenopausal women. Finally, genistein may exert beneficial effects on bone, cardiovasculature, and lipid profiles, all of which are effects characteristic of estrogen (27–29). Taken together, these studies indicate that genistein can behave as an estrogen and can mediate mitogenic effects via the ER.

Estrogens have long been identified as important mitogens in the breast and thus are associated with an increase in breast cancer risk. This is evidenced by the link between reproductive factors, including ages of first menarche, first pregnancy, and menopause, and breast cancer risk (37). This is further supported by studies showing that elevated concentrations of estrogens in serum and urine are associated with increased postmenopausal breast cancer risk (38,39). Additionally, estrogens induce

mitogenic effects in both *in vitro* and *in vivo* models of breast cancer (40). The role of estrogen in this disease is supported by the fact that removal of ovarian estrogens by bilateral ovariectomy (41) or use of tamoxifen (which blocks ER in the mammary gland) (42) significantly reduces breast cancer risk. Because estrogen exposure presumably increases breast cancer risk, evidence showing that genistein acts in an estrogenic fashion is puzzling in light of the *in vitro* reports of genistein as an anticancer agent. To address studies that demonstrate the protective effects of genistein in *in vitro* and *in vivo* breast cancer models, it is important to determine whether genistein has antiestrogenic properties as well.

Possible Antiestrogenic Effects of Genistein

Genistein does not always induce proliferation of ER cells. For example, in some studies genistein exhibits an antiproliferative effect in mammary and uterine tissues (19,43,44). Thus, these data could be interpreted to indicate that genistein is acting as a classical antiestrogen; i.e., it competitively inhibits estrogen's binding to the ER and transactivation of estrogen-responsive genes. However, a more plausible explanation is that genistein inhibits estrogenicity in some other manner. For example, genistein may act in an antiestrogenic fashion through its inhibition of estrogen-metabolizing enzymes. *In vitro* studies in the human breast cancer cell line T47D show the ability of genistein to significantly inhibit the enzyme 17β-hydroxysteroid oxidoreductase type 1 (HSOR-1) (45). HSOR-1 belongs to the family of short-chain alcohol dehydrogenases, which are involved in the metabolism of steroids, antibiotics, and prostaglandins (46). HSOR-1 is necessary for estradiol secretion from the ovaries in premenopausal women. Additionally, it may be essential for the reduction of estrone to estradiol that occurs in the adipose and other tissues (47). Thus, inhibition of this enzyme could lead to decreased total estradiol. *In vitro* studies have also shown that isoflavones can inhibit the aromatase enzyme, which is responsible for the conversion of androgens to estrone in the peripheral (adipose) tissues, although this has not been shown with genistein specifically (48).

The idea of genistein leading to a decrease in estrogenicity through the inhibition of these estrogen-metabolizing enzymes is in contrast to the idea that genistein may increase estrogenicity by inhibiting CYP1A1. On one hand, the inhibition of HSOR-1 could lead to decreased production of estradiol in peripheral tissues and ovarian release of estradiol, thus resulting in a decrease in

total estradiol, whereas inhibition of CYP1A1 could result in the opposite, leading to a buildup of total estradiol levels. It is not clear, therefore, what the net effect of genistein would be on overall estrogenicity (Figure 1). Furthermore, in addition to genistein's ability to inhibit the enzymatic activity of HSOR-1 and CYP1A1, genistein may affect these estrogen-metabolizing enzymes in another fashion. It is possible that the disruptive effects of genistein on the hypothalamic/pituitary/gonadal axis could lead to an upregulation of one or more of these enzymes, further complicating the issue of the overall estrogenic effect of genistein. However, depending on which, if any, of these enzymes would be upregulated in response to a perturbation of the hypothalamic/pituitary/gonadal axis, it is possible that the overall effect of genistein could be either an increase or decrease in estrogenicity. Thus, the overall estrogenic/antiestrogenic effects of genistein, as a result of inhibiting the activity of estrogen-metabolizing enzymes, are unclear and warrant further study.

In support of the possibility that genistein may have antiestrogenic effects by reducing circulating estrogen levels, there is some evidence that the consumption of high levels of soy products decreases plasma estradiol concentrations in premenopausal women (49,50). These women also had lengthened menstrual cycles (23). However, in some studies serum estradiol levels were not changed (35,36,51,52) or were increased in women supplemented with soy (53). Postmenopausal women did not show any of these effects. Perhaps the contrasting findings can be partially explained by the observation that genistein reduces gonadotrophin hormone-induced release of luteinizing hormone and follicular stimulating hormone (54). This in turn would initially lead to a reduction in estrogen production from the ovaries and eventually perhaps to an increase through a negative feedback mechanism acting on the hypothalamic/pituitary/gonadal axis (Figure 1). However, because genistein also interferes with HSOR-1, resulting in reduced estradiol production, and CYP1A1, resulting in increased estradiol levels, the net result may be either reduced or increased circulating estradiol levels, perhaps depending on the presence of additional simultaneous estrogenic stimuli (e.g., dietary fat).

Regardless of an effect on the concentration of circulating estradiol, genistein may be able to reduce the bioavailability of estradiol by increasing the synthesis of sex-hormone-binding globulin (SHBG) through stabilization of SHBG mRNA in a manner analogous to estradiol (55). SHBG binds and sequesters hormones, thereby reducing the concentration of bioavailable or free hormone.

However, in the presence of genistein, increased levels of SHBG may not necessarily lead to reduced estrogenicity. It is possible that when estradiol is bound to SHBG, genistein is free to bind to ER, resulting in transactivation of estrogen-responsive genes. Furthermore, given the structural similarities of genistein and endogenous estrogens, it is feasible that genistein could bind to SHBG and displace sequestered estrogens, thus effectively increasing the concentration of free estrogens in the body. However, studies by Baker et al. (56) and Martin et al. (30) show that genistein's binding affinities for rat alpha-fetoprotein (another member of the steroid-hormone-binding family of proteins) and human SHBG are significantly less than that of estradiol. This evidence suggests that genistein is probably not acting to displace endogenous estrogen from its binding proteins in humans and rodents (56). Thus, these data as a whole demonstrate that genistein may be a modulator of bioavailable estrogens. However, given its potential estrogenic activity, the cumulative effects of this reduction in endogenous estrogens, either by inhibiting metabolism or increasing sequestration, may not be significant.

Another possible antiestrogenic effect of genistein may be its ability to alter the kinetics of receptor translocation. Studies in the MCF-7 human breast cancer cell line indicate that genistein-bound ER may translocate to the nucleus more slowly than estrogen-bound ER, thus resulting in an antiestrogen-like effect of diminished signal transduction (30). However, this phenomenon has not been widely studied.

It is clear that *in vitro* genistein competes with estradiol for receptor binding (57). However, because genistein binds to both the classic ER-α and the novel ER-β receptor subtypes with relative binding affinities of roughly 20- and 5-fold less than that of estradiol, respectively (58), it is possible that genistein may not effectively compete with estradiol for ER binding *in vivo*. Furthermore, ER-α and ER-β, when bound to estradiol, appear to exert opposite effects on the AP1 transcription factor; i.e., estradiol leads to transcriptional activation when complexed with ER-α, whereas estradiol represses transcription when coupled with ER-β (59). There is some evidence to indicate that activation of ER-β inhibits cellular proliferation. For example, several antiestrogens behave as potent transcriptional activators when bound to ER-β at an AP1 site (59), and this may explain why an inhibition of cell growth occurs. Because genistein preferentially binds to ER-β, it may induce antiestrogenic effects through this receptor isoform.

In summary, genistein may have antiestrogenic effects through binding preferentially to ER-β, by inhibiting the activity of enzymes that participate in estrogen metabolism, or by affecting the hypothalamus in an estrogenic manner. The two latter effects can further lead to reduced circulating estrogen levels and increased menstrual cycle length. These possibilities are illustrated in Figure 1 and summarized in Table 2.

Genistein—Anticancer or Cancer-Promoting Compound?

Clearly, based on the findings of studies addressing the estrogenicity and antiestrogenicity of genistein, it cannot be concluded whether the net result of genistein consumption will be a proliferative or antiproliferative effect on breast cells. Many reports in the literature indicate that genistein is an inhibitor rather than a stimulator of mammary carcinogenesis. These reports consist of studies conducted in human breast cancer cells in which genistein inhibits cell growth (20), animal studies in which genistein inhibits initiation or promotion of carcinogen-induced mammary tumorigenesis (44,60), and epidemiologic studies in which high soy intake is linked to reduced breast cancer risk (61,62). However, the literature also contains many studies that have produced contrasting data.

In vitro *studies*. Studies conducted in human breast cancer cell lines indicate that genistein both inhibits and stimulates proliferation of these cells. For example, Hsieh et al. (18) observed a mitogenic effect of genistein at low doses (0.01–1 μM) and an antiproliferative effect at higher doses (> 10 μM). The results of this study are consistent with the data of Wang et al. (20), in which the growth of MCF-7 cells was stimulated and then inhibited by genistein in a dose-dependent manner. Thus, at doses of ≤ 1 μM, genistein appears to stimulate the growth of ER+ breast cancer cells (18,20). These doses correspond to the human (physiologic) exposure level because most Asians or Caucasians that consume a high soy diet have serum genistein levels of < 1 μM. Conversely, doses > 10 μM genistein inhibit the growth of both ER+ and ER-negative (ER–) breast cancer cells (20). This strongly implicates that the mechanisms of inhibition of cell proliferation by pharmacologic doses of genistein occur independently of the ER. It is also apparent that at physiologic exposure levels,
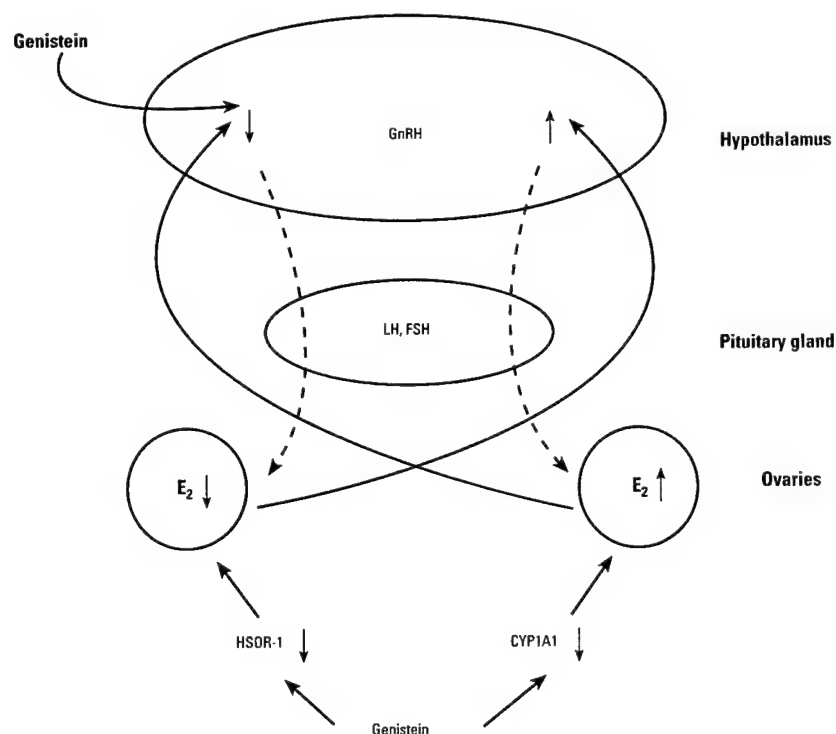

Figure 1. Potential pathways leading to estrogenic/antiestrogenic effects of genistein.

Table 2. Mechanisms through which genistein could reduce estrogenicity.

Observation	Reference
Inhibition of enzymes that metabolize estrogens	(45,48)
Reduction in circulating estradiol levels	(49,50)
Lengthened menstrual cycle	(23)
Stimulation of sex-hormone-binding protein	(55)
Preferential bindings to ER-β, leading to inhibition of AP-1 transcription	(58) (59)

Some of these findings are opposed or unconfirmed by other data.

it is more likely that breast cell growth is stimulated rather than inhibited by genistein.

Animal data. Most animal studies are supportive of the hypothesis that genistein or consumption of soy protein inhibits mammary tumor promotion (*63–66*). These findings were obtained from studies in intact rats and mice with functional ovaries that were exposed to carcinogens to initiate tumor formation. However, some studies show an ability of genistein to increase mammary tumorigenesis. Hsieh et al. (*18*) examined the effects of genistein exposure on ovariectomized athymic mice. Genistein, when administered through the diet at a dose of 750 ppm, enhanced epithelial proliferation in the mammary gland as well as the growth of MCF-7 cell (ER+) tumors *in vivo*. These *in vivo* results are particularly compelling because they were obtained in ovariectomized mice, which may represent a model for breast cancer development in postmenopausal women. Thus genistein, albeit a weak estrogen, could have mitogenic effects on mammary tissue when serum estrogen levels are low, such as in postmenopausal women.

Human data. The estrogenic effects of genistein/soy are not only seen in *in vitro* studies or in animal models. Petrakis et al. (*23*) found that 5 months of daily intake of 38 g soy protein isolate containing 38 mg genistein increased the yield of nipple aspirate fluid (NAF) and the appearance of hyperplastic epithelial cells in premenopausal women. There were no significant changes observed in postmenopausal women (*23*). Previous studies showed a reduced total volume, a lighter color, and less atypical epithelial cells in the NAF obtained from women with low breast cancer risk (i.e., Chinese and Japanese women) compared to women with high breast cancer risk (*67–69*). These studies suggest a correlation between volume, color, and cytology of NAF with breast cancer risk. In another study, 14-day daily intake of 60 g soy supplement containing 45 mg isoflavones significantly increased the proliferative rate of breast lobular epithelium in premenopausal women (*24*). Both of these studies indicate that soy/genistein has an estrogenic effect on the human breast. Furthermore, the results obtained in the soy/NAF/breast proliferation studies would seem to suggest that the genistein in soy is not acting in a protective manner in premenopausal women and may have no effect in postmenopausal women.

Caution must be exercised when interpreting the data of these two studies that apparently indicate an estrogenic effect of soy on the human breast. In particular, the Petrakis et al. (*23*) study contains several potential confounding factors. First, menstrual cycling was not controlled for in this

study, and thus NAFs were not obtained at the same phase of the menstrual cycle. However, this may not have affected the results because menstrual cycle has not been found to affect either the hormonal or cytologic content of NAF (*70*). Second, some women in the study were exposed either to oral contraceptives or hormone replacement therapy, and this could have interacted with the effects of soy on NAF. Third, all women previously yielded NAF, and because yielders are at a higher risk to develop breast cancer than nonyielders (*67,68*), study subjects may have been particularly sensitive to the estrogenic effects of genistein in soy. Finally, it is possible that other components of a Western diet may have interacted with soy administration, producing a different outcome than if study subjects had been Asian women consuming an Asian diet.

The epidemiologic evidence supporting the idea that high soy consumption protects against breast cancer is also inconsistent (*61*). Three studies suggest that soy intake is associated with lower breast cancer risk. A study in Singaporean women found that high soy intake is associated with a lower breast cancer risk among premenopausal but not postmenopausal women (*62*). A study in Asian-American women living in the West indicated that breast cancer risk decreases with increasing frequency of tofu (bean curd) intake in both pre- and postmenopausal women (*71*). Finally, a study that measured urinary excretion levels of phytoestrogens reported that a high excretion of isoflavones (genistein was not included) was associated with a substantial reduction in breast cancer risk (*72*). A similar but more recent study did not find significant differences in soy protein intake or urinary excretion levels of daidzein or genistein between breast cancer cases and their controls in Shanghai; however, total isoflavonoid levels in urine were lower in the breast cancer cases (*73*). Four other studies also suggest that soy consumption is not associated with a reduced risk of breast cancer (*74–76*). These significant inconsistencies may reflect differences in the end points used for soy/genistein intake (consumption of tofu or miso, or serum isoflavone concentrations). They may also suggest that high intake of genistein is not the key factor behind low breast cancer incidence in Asian countries. We recently performed a meta-analysis of all the epidemiologic studies currently available. The results indicate that high soy intake might reduce the risk of developing premenopausal breast cancer, but has no effect on postmenopausal breast cancer risk (*77*).

Conclusions from in vitro *and* in vivo *data.* There appears to be a great disparity among the findings of both animal and

human studies. Is genistein an anticancer or a cancer-promoting agent? The answer may very well be both. One of the most convincing explanations for the duality of this compound lies in the experimental design—specifically dosage. It is well documented that the classical antiestrogen tamoxifen has agonist properties when administered at low doses and antagonistic properties when administered at higher doses (*78*). Thus studies showing antiproliferative effects using genistein at high doses (> 1 μM) could show similar antiestrogenic actions such as those with tamoxifen. Indeed, many *in vitro* studies conducted using human breast cancer cell lines indicate a biphasic effect of genistein. Interestingly, a study by Anderson et al. (*27*) showed that at low doses, genistein exerted estrogen-like beneficial effects on bone tissue in ovariectomized rats, whereas at high concentrations these estrogenic effects were not seen. Given the potentially biphasic effects of genistein, it is important to determine what the relevance of these concentrations is to women on soy-supplemented diets. Reports in women consuming large amounts of soy products indicate concentrations of up to 0.25 mg/kg genistein in the plasma and urine (*10*). It appears from *in vitro* and *in vivo* animal studies that pharmacologic doses may be required for the breast cancer preventative effects seen with genistein. It is possible that in humans a diet containing foods high in genistein will never reach the levels that are able to effectively inhibit mammary tumorigenesis *in vitro* and in animal models. However, this conclusion is contradicted by our meta-analysis, which showed a protective effect of high soy intake among premenopausal women (*77*), and in animal studies in which rats were given a physiologic dose of genistein prepubertally, resulting in reduced mammary tumor incidence (*79*).

Genistein and Nonestrogenic Pathways of Action

The antiproliferative effects seen with pharmacologic doses of genistein are unlikely to be mediated by the ER. Instead, they are probably due to other biological effects of genistein. For example, genistein is a specific inhibitor of protein tyrosine kinase (PTK) (*11*). Genistein induces a reversible G_2M cell cycle arrest, which may be related to its ability to inhibit PTK (*80,81*). Other important mechanisms by which genistein may exert its antiproliferative effects are through its ability to inhibit both topoisomerase II and angiogenesis (*12,13*). Additionally, it has recently been suggested that the growth inhibitory effects of genistein may be due to modulations in transforming growth factor-β signal transduction (*82*).

PTKs regulate a number of growth factor receptor signal transduction pathways, which can become oncogenic when altered. Therefore, a potent inhibitor of PTK could counteract this oncogenic activity. One widely studied PTK-associated growth-promoting pathway is the epidermal growth factor pathway. Increased expression of the epidermal growth factor receptor (EGFR) in breast cancer cells has been associated with accelerated growth and metastasis and is an indicator of poor prognosis (*83*). EGFR contains a PTK domain and upon activation and dimerization of the receptor, phosphorylation of the PTK domain occurs. This results in signaling events to downstream effector molecules, ultimately leading to an inhibition of apoptosis. It has been postulated that genistein's ability to inhibit the PTK of EGFR (or other potentially oncogenic proteins) may be an important mechanism mediating its observed antiproliferative effects in breast cancer. In support of this, a number of studies have demonstrated genistein's ability to inhibit both PTK and the proliferation of a number of ER+ and ER– breast cancer cell lines (*84,85*). An indication that genistein-induced inhibition of PTK may be independent of ER-mediated functions was shown by Schultze-Mosgau et al. (*85*). The authors demonstrated that pharmacologic doses of genistein inhibit the PTK-dependent transcription of c-*fos* and subsequent cellular proliferation in an ER- human breast cancer cell line. Thus, the antiproliferative effects appear to be due to an inhibition of PTK rather than an inhibition of ER signaling. Additionally, a study by Uckun et al. (*86*) showed that targeting nanomolar concentrations of genistein to the EGFR–PTK complex by means of an EGF–genistein conjugate resulted in a rapid apoptotic effect and inhibition of *in vitro* clonogenicity in human breast cancer cells. Similar results were seen when targeting the Src family of PTK with an anti-CD19 antibody–genistein conjugate (*87*). As with the EGF–genistein conjugate, this was effective at increasing apoptosis and decreasing cell growth at nanomolar concentrations.

Genistein's ability to inhibit tyrosine phosphorylation not only allows for an inhibition of a proliferation of cancer cells, but it may also lead to an inhibition of metastasis. It has been suggested that tyrosine phosphorylation of membrane proteins plays a critical role in the mediation of degradation of the extracellular matrix, thus allowing for cellular invasion (*88*). In a recent study, Connolly et al. (*89*) attempted to reverse the metastasis promoting effects of *n*-6 polyunsaturated fatty acids (PUFAs) in intact nude mice transplanted with ER– human breast cancer cells (MDA-MB-435) by feeding the animals soy protein. They found that soy increased, rather than decreased, the size of both primary tumors and lung micrometastases in the high *n*-6 PUFA group. However, the number of macrometastases was significantly reduced by soy. These results suggest that genistein/soy may promote mammary tumor growth in the nude mouse model both in ER+ (*18*) and ER– tumor cells (*89*) but has both inhibitory and stimulatory effects on various aspects of metastasis (*89*). In addition, Li et al. (*90*) demonstrated that genistein inhibits the secretion of matrix metalloproteinase (proteinases which have been implicated in invasion and metastasis) in MDA-MB-435 breast cancer cells.

It is important to note that pharmacologic doses are required for all of these non-ER-mediated effects of genistein [concentration that inhibits cell growth by 50% (IC_{50}) > 1 μM], with the exception of the genistein conjugates used by Uckun et al. (*86,87*). Interestingly, Peterson et al. (*84*) demonstrated that although genistein can induce growth inhibition of a number of breast cancer cell lines at pharmacologic doses (IC_{50} 2.3–20 μg/mL), these doses do not correlate with PTK inhibition. Doses of 50 μg/mL genistein were required to significantly inhibit EGFR tyrosine phosphorylation. Schultze-Mosgau et al. (*85*) showed similar findings for EGFR in MDA-MB-468 breast cancer cells. Genistein was able to inhibit the growth of these cells at an IC_{50} of < 10 μM, whereas PTK was inhibited at doses of 60 μM (*86*). Additionally, Peterson and Barnes (*84*) showed that doses up to 20 μg/mL genistein were insufficient to inhibit the tyrosine phosphorylation of other PTKs such as phospholipase Cγ and Raf, whereas doses up to 50 μg/mL did not inhibit tyrosine phosphorylation of MAPK or PI3K. This is further supported by Koroma et al. (*80*), who reported that in bovine aortic endothelial cells, genistein doses of > 300 μM were required to inhibit PTK, whereas growth was inhibited at concentrations of < 30 μM. Thus, these data strongly suggest that the antiproliferative effects of genistein are not mediated through inhibition of PTK.

However, although genistein does not appear to be inhibiting EGFR (or other PTKs that have been studied) at physiologic doses, or even at pharmacologic doses associated with growth inhibition, this does not preclude the possibility that genistein mediates its growth-inhibitory effects through the inhibition of other PTK-dependent pathways. It is possible that at lower pharmacologic doses (those associated with genistein's growth inhibitory effects) genistein targets an as yet unidentified kinase or a kinase which has not been examined within this context. Additionally, kinase inhibitors specific for a particular kinase can inhibit other kinases when administered at extremely high doses. This may be due to the similarities in structure of these inhibitors, given that many are designed to target the adenosine triphosphate (ATP) binding site of PTKs. The possibility of genistein mediating its growth-suppressive effects through the inhibition of an unknown kinase at lower pharmacologic doses and nonspecifically inhibiting other kinases at high doses could explain the observed lack of correlation between growth inhibition at lower doses and PTK inhibition of EGFR by higher dose genistein.

The Uckun studies (*86,87*) targeting conjugated genistein into direct association with PTK raise an important possibility. In these studies conjugated genistein was able to inhibit PTK at nanomolar concentrations; in the same experiments, genistein alone was unable to achieve this even at micromolar concentrations (> 10 μM for EGFR inhibition). However, when directed to PTK genistein is effective at physiologic concentrations in the nanomolar range. Proposed explanations for the efficacy of these genistein conjugates at nanomolar concentrations are increased delivery of genistein, direct contact with PTK, and localization in close proximity to ATP binding domains of PTK, thus possibly increasing the binding constant. Nevertheless, despite a lack of understanding of the exact mechanism of genistein's antiproliferative action, it is clear that at pharmacologic doses genistein may be an important inhibitor of breast cancer cell growth and may alter metastatic properties. Additionally, it is possible that even at physiologic concentrations genistein has the potential to exert anticancer properties through inhibition of PTK, but lacks the ability to effectively reach such target molecules.

Timing of Genistein Exposure and Mammary Tumorigenesis

In addition to dose, timing of administration may explain the apparent dual effects of genistein. Throughout the life span, estrogens increase mammary cell proliferation, but depending on the overall hormonal environment, estrogens also activate expression of other factors that could induce differentiation or affect mammary growth by other means. Thus, estrogens can have a different impact on the breast if the exposure occurs *in utero*; during childhood, puberty, or pregnancy; premenopausally; or during postmenopause (*91*). There is evidence that genistein also has different effects on the breast depending on the timing of exposure.

We examined the effects of exposing pregnant rats to genistein (at doses ranging from 20 to 300 μg) on mammary gland development and tumorigenesis among the

offspring (22,92). The rationale for this study was based on the fact that mice and rats exposed to estradiol or other estrogenic compounds *in utero* only, i.e., time of exposure was restricted to the *in utero* period, exhibit an increase in mammary tumorigenesis (93,94). Furthermore, epidemiologic data suggest that high estrogen levels during pregnancy increase breast cancer risk among daughters (95–97). Our results indicate that *in utero* exposure to genistein through a pregnant mother increases the risk to develop 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced mammary tumorigenesis in rats but does not increase the number of tumors per rat (multiplicity) (92). Thus *in utero* genistein may act as an estrogen in the mammary gland. Interestingly, a recent study found that the free fraction of estradiol in fetal cord serum is 0.05% of that in adult human serum (98). Nagel et al. (98) therefore postulated that there may be an undefined mechanism limiting the uptake of endogenous estrogens. They go on to suggest that phytoestrogens may not be limited by this uptake mechanism, which could result in increased exposure to these exogenous estrogens during prenatal life. This is speculative, however, and needs to be further substantiated.

The mechanism mediating the effects of *in utero* estrogen/genistein exposure on mammary tumorigenesis may be related to changes in mammary gland morphology. In the mammary gland, terminal end buds (TEBs) are the least mature ductal structures, containing multipotent stem cells, and are the most susceptible to carcinogens. With maturation, TEBs either differentiate into alveolar buds (which comprise type I and II lobules) or regress to terminal ducts. During pregnancy, lobules I and II further mature into type III lobules. In rats, and perhaps also in humans, more differentiated lobular structures give rise only to benign tumors (99). Our data indicate that in animals exposed to estrogens or genistein *in utero*, the number of TEBs in the mammary gland is increased and remains increased, and the number of terminal ducts and differentiated alveolar buds is reduced, when compared to animals exposed to vehicle control *in utero* (22).

The findings indicating that *in utero* exposure to genistein increases mammary tumorigenesis in rats are in sharp contrast to findings in Asian women. These women consume high levels of soy products, including during pregnancy, and their newborns have high plasma levels of phytoestrogens at birth similar to their mothers' levels (100). However, breast cancer risk is low among Asian women. One explanation may be that Asian women are exposed to high levels of phytoestrogens throughout their lives,

whereas in our study rats received genistein only *in utero*. Another explanation could be that soy has many other components in addition to genistein, and these components may antagonize the estrogenic effects of genistein *in utero*. This interpretation is supported by our unpublished study (101) in which no changes in DMBA-induced mammary tumorigenesis were noted in offspring of rat dams who consumed varying levels of soy protein during pregnancy.

The period when the breast is particularly vulnerable to the effects of carcinogens is between puberty and a first full-term pregnancy. During this time, there are a high percentage of TEBs and many actively proliferating cells in the breast. We (79) and others (60,102,103) have studied the effects of prepubertal genistein exposure on mammary tumorigenesis induced by DMBA. Studies by Lamartiniere et al. (60), Murrill et al. (102), and Brown et al. (103) demonstrated that postpartum (days 2, 4, and 6) or prepubertal (days 16, 18, and 20) treatment of rats with 5 mg genistein resulted in increased latency and reduced the incidence and multiplicity of breast tumors. We (79) recently replicated these findings by administering 20 µg genistein between postnatal days 7 and 21; this dose is closer to the human exposure range. At day 21, the genistein-exposed animals had a higher percentage of TEBs and increased cellular proliferation compared to control animals, but by day 50 the TEBs had differentiated to lobular structures, which were not susceptible to malignant growth, and the glands exhibited less cellular proliferation (60,102). Thus, genistein administered after birth but before the onset of puberty may, in the long term, have a differentiating effect on mammary gland ductal structures and may be chemopreventive (60). It is also important to point out that prepubertal genistein administration did not alter puberty onset, although estradiol exposure during the same time period effectively advances puberty. The results of studies in which genistein was administered *in utero* or before puberty indicate that these exposures can have significant but opposing effects on the normal development of TEBs in the mammary gland and influence its susceptibility to carcinogenesis. Fritz et al. (104) recently conducted a study in which rats were exposed via diet to genistein from conception (and throughout fetal life) to postpartum day 21. This perinatal exposure significantly reduced the multiplicity (number of tumors per animal) of DMBA-induced mammary tumors but did not affect the proportion of animals per group who developed tumors (tumor incidence). Thus, it appears that some of the adverse effects of *in utero* genistein exposure on mammary

tumorigenesis can be partially reversed by prepubertal exposure to the same phytoestrogen.

It is difficult to conceptualize why genistein would have different effects on breast cancer risk based on the timing of exposure. Do these findings suggest that genistein is estrogenic *in utero* and antiestrogenic during adolescence? In addition, is genistein also antiestrogenic during the reproductive years and estrogenic again postmenopausally? It is more likely that genistein is always acting as an estrogen if the level of exposure is maintained at a level low enough as to stimulate only the ER. The differentiating effect on the mammary gland with prepubertal genistein exposure also occurs after prepubertal estradiol exposure. For example, animal studies indicate that neonatal and postpubertal exposure to estrogens reduces subsequent mammary tumorigenesis (105,106). Furthermore, in human studies, high fat intake or high body mass index at puberty, both of which increase availability of nongonadal estrogens, are linked to reduced (not increased) breast cancer risk (107,108). These data suggest that high prepubertal estrogen levels may effectively protect the breast from malignant transformation, perhaps by inducing early breast differentiation.

During the reproductive years, genistein increases mammary gland proliferation, as is evident in two human studies (23,24). Animal studies also show that genistein induces proliferation of the mammary epithelial structures (18). However, there is no evidence that genistein increases breast cancer risk in premenopausal women; it may modestly reduce it (77). Animal data suggest that genistein may promote breast cancer growth in ovariectomized mice (18); i.e., in a postmenopausal breast cancer model. The apparent difference in the effects of genistein on breast cancer risk premenopausally and postmenopausally may be explained by the fact that the breasts of older women are more likely to contain malignant cells as compared to the breasts of younger women. Although the proliferative effects of genistein on postmenopausal women have not been extensively studied, it is possible that genistein induces proliferation of mammary cells in both pre- and postmenopausal women. However, in postmenopausal women, who are more likely to have accumulated malignancies than their younger counterparts, genistein may stimulate the proliferation of these malignant cells leading to the formation of breast cancer. Therefore, genistein may not be antiestrogenic premenopausally, but rather, the mammary gland of younger women may be less fertile ground for the development of cancer.

In conclusion, the differential effects of genistein on breast cancer risk throughout

life from *in utero* to the postmenopausal period appear similar to those of endogenous estrogens, further supporting the role of genistein as an estrogenic compound.

Conclusion

Based on data in the literature, it appears that genistein can act as both an estrogen and an antiproliferative agent. These effects may be both dose and tissue dependent. This is in agreement with data from studies with other estrogenic compounds such as tamoxifen (a partial estrogen receptor agonist) and diethylstilbestrol (a potent ER agonist). Additionally, the timing of exposure may be critical in determining the carcinogenic/anticarcinogenic potential of genistein. Gonadal and placental estrogen production varies dramatically during a woman's life span, as does the production of other factors that regulate the breast. It is plausible that genistein has different effects on the breast in the presence of high estrogen levels (such as during pregnancy), moderate levels (such as during premenopausal life), and low levels (such as during childhood and postmenopause).

It is clear from epidemiologic data that Asian women living in Asia (where a diet high in soy is consumed) have a decreased risk for breast cancer. One possible explanation for the apparent lack of tumor-promoting effects of genistein in Asian populations is that a lifetime exposure to genistein may have a protective effect. It is also possible that soy contains other factors besides genistein that oppose the estrogenic effects of genistein and actually reduce breast cancer risk. Additionally, other environmental or lifestyle factors may be more related to the low breast cancer risk seen in this population than soy/genistein consumption.

Although the evidence of the range of genistein's effects is far from conclusive, it is tempting for some in the scientific community to tout genistein as a potential chemopreventive agent or alternative to hormone replacement therapy. However, studies indicating a potential cancer-promoting effect of genistein should not be taken lightly. Further studies must be done before the true scope of genistein's actions can be understood. Given the recent U.S. Food and Drug Administration approval of an over-the-counter soy supplement and recent media reports touting this as an alternative to hormone replacement therapy, the need for a clearer understanding of the potential cancer-promoting effects of genistein is paramount. Fortunately, genistein continues to be an active area of research interest, and therefore an explanation for the dual nature of genistein may not be too far away.

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Appendix B. Figures

MANUSCRIPT: CABANES A, OLIVO S, DE ASSIS S, GUSTAFSSON J-A, <u>HILAKIVI-CLARKE L</u> Prepubertal estradiol exposure increases estrogen receptor beta levels in the mammary gland and reduces 7,12-dimethylbenz[a]-antracene-induced mammary tumorigenesis in rats. A manuscript in preparation, 2001.

FIGURE LEGENDS

Figure 2. The proportion of female rats exposed to 10 μg E2 (n=20) or vehicle (n=22) between postnatal days 7 and 20, that developed DMBA-induced (administered on day 47) mammary tumors. Tumor incidence was significantly lower in the E2 exposed rats (Survival analysis: $p < 0.002$).

<u>Finding</u>: Prepubertal E2 exposure reduces later mammary tumor incidence.

Figure 3. Changes in mammary epithelial tree in rats (n=5-6 animals per group and age) exposed to 10 μg E2 during prepuberty, assessed in wholemounts obtained at 3, 8, 16 and 25 weeks. Only the animals whose glands were obtained on week 25 were also exposed to DMBA. Total number of TEBs was counted, and the density of epithelial tree and alveolar buds and lobules were estimated using a visual scale ranging from 0=absent to 5=numerous. Statistically significant difference: * $p < 0.05$, ** $p < 0.01$

<u>Finding</u>: Prepubertal E2 exposure reduces mammary epithelial density and number of terminal end buds, and increases the density of alveolar buds.

Figure 5. *Quantitative data on ER-β protein expression in the mammary gland*. ER-β protein levels in the mammary glands of 3, 8 and 16-week-old rats exposed to either vehicle (V) or 10 μg of estradiol (E2) during prepuberty: Fifty μg of protein were electrophoresed under reducing conditions in 8%Tris-Glycine gels and transferred into a nitrocellulose membrane. ER-β protein was detected with a polyclonal antibody obtained from Dr Gustafsson (LDB; 1:4000 dilution). Only one reactive band was observed in mammary gland with this antibody, that migrated approximately at 61-62 kDa. Statistically significant difference: ** $p < 0.01$.

<u>Finding</u>: Prepubertal E2 exposure significantly elevates ER-β levels in the mammary glands of 8 and 16-week-old rats.
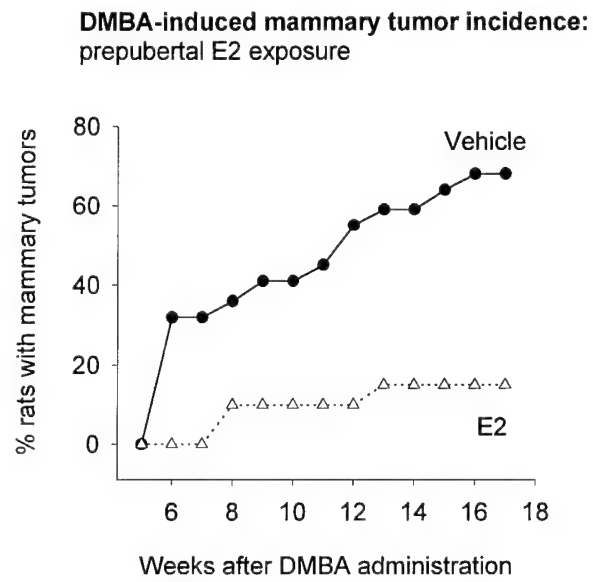
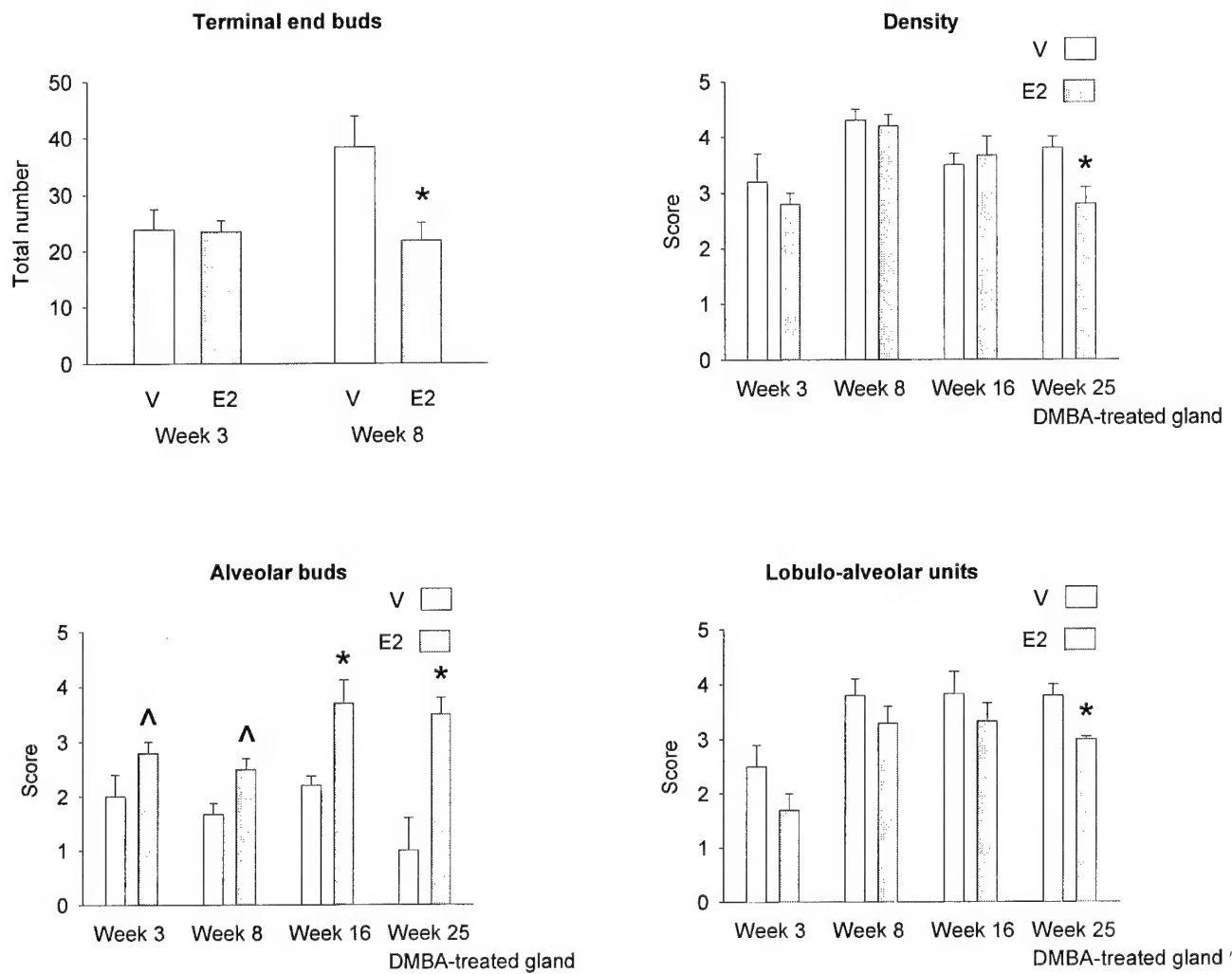**DMBA-induced mammary tumor incidence:**
prepubertal E2 exposure

Figure 2.
Cabanes et al.

Figure 3.
Cabanes et al.

ER-beta protein levels

Figure 5.
Cabanes et al.

3-week-old rats

8-week-old rats

16-week-old rats
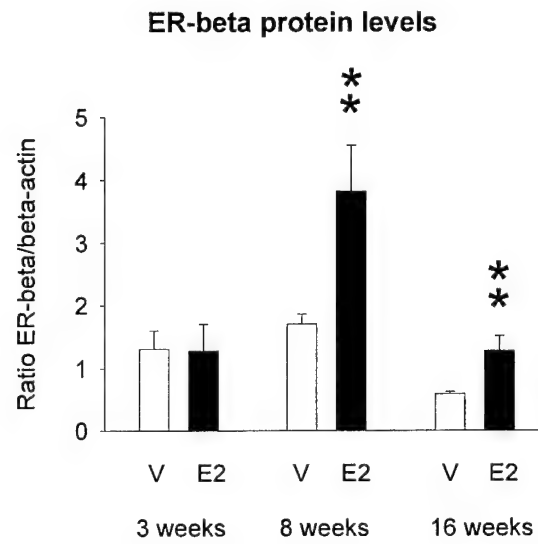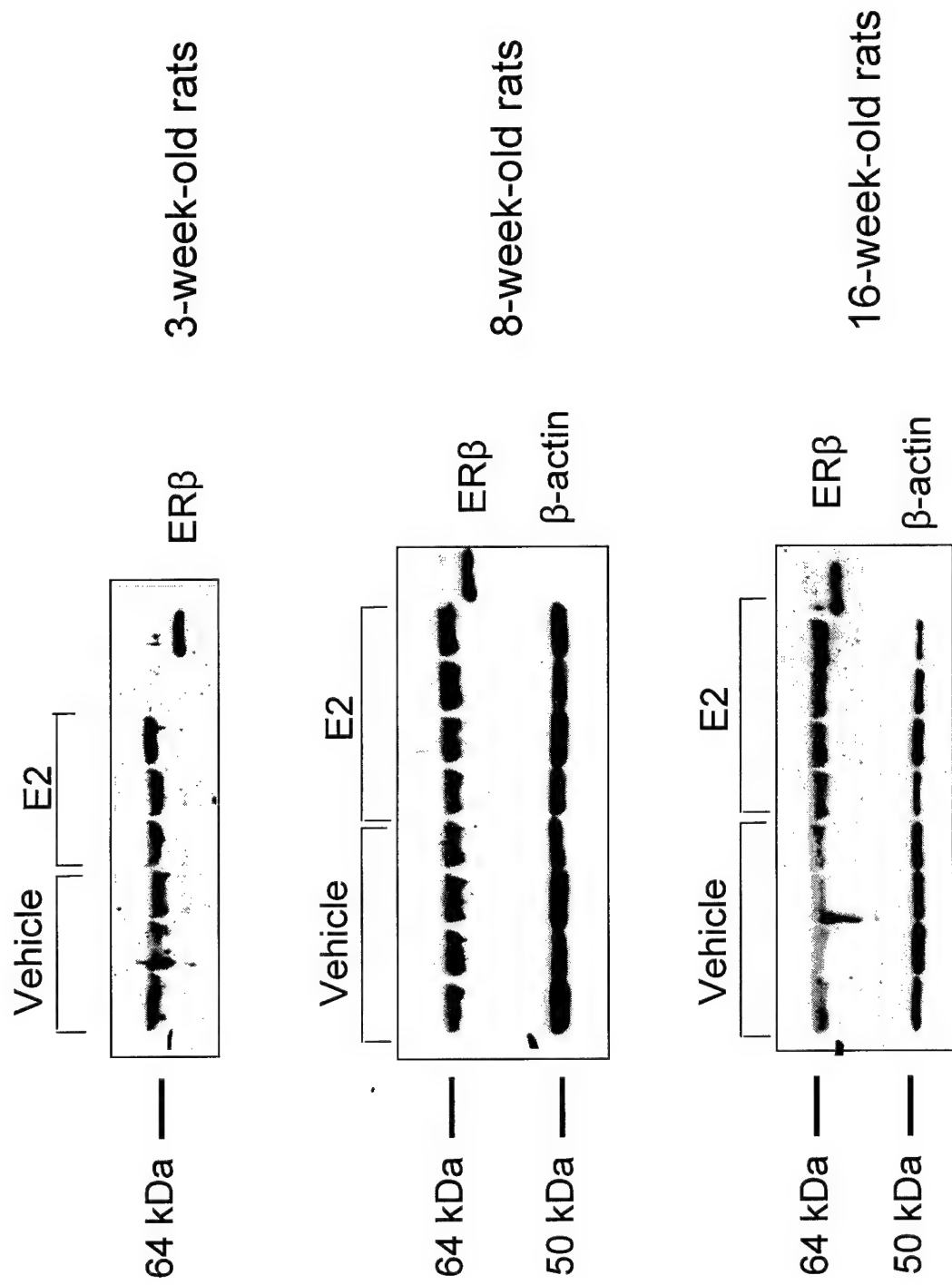
MANUSCRIPT: <u>HILAKIVI-CLARKE L</u>, CHO E, CABANES A, DEASSIS S, OLIVO S, HELFERICH W, LIPPMAN ME, CLARKE R. Modulation of pregnancy estrogen levels by maternal dietary exposure to soy isolate or n-3 polyunsaturated fatty acid and breast cancer risk among female rat offspring. A manuscript in preparation, 2001.

FIGURE LEGENDS

Figure 1. Serum E2 levels on gestation day 18 or 19 in rats fed (A) diets containing high-fat corn oil or low- or high-fat menhaden oil, or (B) diets containing low (15 mg/kg diet), medium (150 mg) or high (300 mg) genistein levels in soy isolate. * Significantly different from the high-fat corn oil group; one-way ANOVA: $p < 0.05$.

<u>Finding</u>: Maternal dietary exposure to n-3 PUFA diet significantly increases pregnancy estradiol levels, and maternal dietary exposure to high genistein levels in soy isolate non-signficantly increases the levels.

Figure 2. Serum E2 levels on postnatal week 3 or 8 in female offspring of dams fed (A) diets containing low- or high-fat n-6 PUFA corn oil or n-3 PUFA menhaden oil, or (B) diets containing low (15 mg/kg diet), medium (150 mg) or high (300 mg) genistein levels in soy isolate. (A): * Significantly different from the low-fat n-6 PUFA group; one-way ANOVA: $p < 0.05$; (B): * Significantly different from the low genistein group; one-way ANOVA: $p < 0.05$.

<u>Finding</u>: Maternal dietary exposure to high-fat n-3 PUFA diet or high genistein levels in soy isolate during pregnancy significantly reduces offspring's estradiol levels.

Figure 3. Total number of terminal end buds (TEBs) or relative density of lobules on postnatal week 3 or 8 in female offspring of dams fed (A) diets containing low- or high-fat n-6 PUFA corn oil or n-3 PUFA menhaden oil, or (B) diets containing low (15 mg/kg diet), medium (150 mg) or high (300 mg) genistein levels in soy isolate. (A): * Significantly different from the other three groups; one-way ANOVA: $p < 0.05$; (B): * Significantly different from the low genistein group; one-way ANOVA: $p < 0.05$.

<u>Finding</u>: Maternal dietary exposure to n-3 PUFA diet significantly reduces the number of terminal end buds in offspring's mammary gland. Maternal dietary exposure to high genistein levels in soy isolate increases the number of terminal end buds and reduces the density of lobules.

Figure 4. Proportion of rats that developed at least one mammary tumor following an exposure to DMBA. Tumor incidence was determined weekly, between weeks 6 and 17. Results are shown for offspring whose mothers were fed (A) diets containing low- or high-fat menhaden or corn oil, or (B) diets containing low (15 mg/kg diet), medium (150 mg) or high (300 mg) amounts of genistein in soy isolate. (A): Tumor incidence was significantly lower in the high n-3 PUFA offspring, when compared to high n-6 PUFA offspring (Survival analysis: $p < 0.00^*$).

<u>Finding</u>: Maternal dietary exposure to high-fat n-3 PUFA diet significantly reduces offspring's mammary tumor incidence, while maternal dietary exposure to high genistein levels in soy isolate has no effect.

19

Serum E2 levels in fat-fed dams:
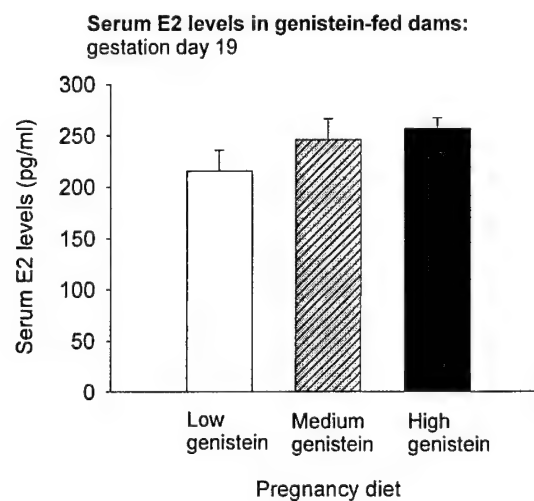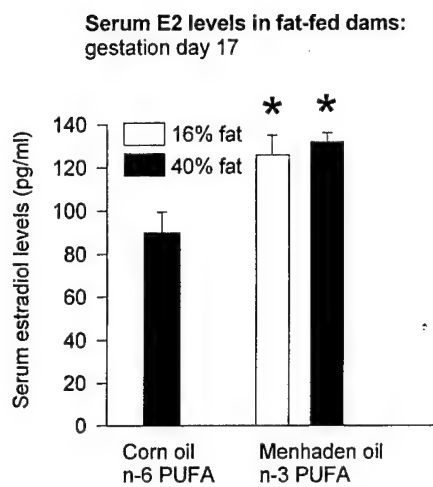gestation day 17

Serum E2 levels in genistein-fed dams:
gestation day 19

Figure 1.

Hilakivi-Clarke et al.

Serum E2 levels:
offspring of mothers fed fat diets during pregnancy
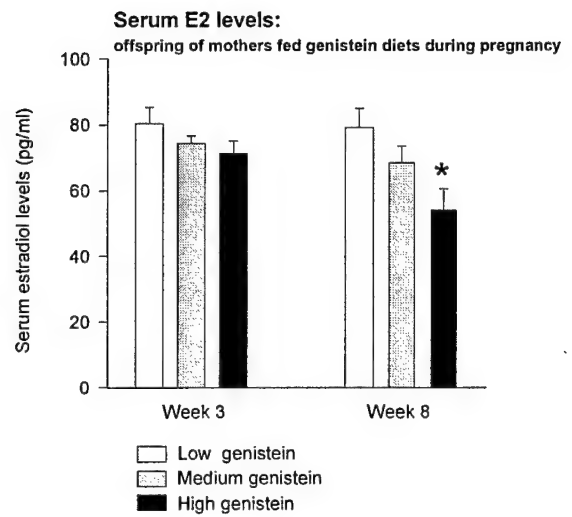
Serum E2 levels:
offspring of mothers fed genistein diets during pregnancy
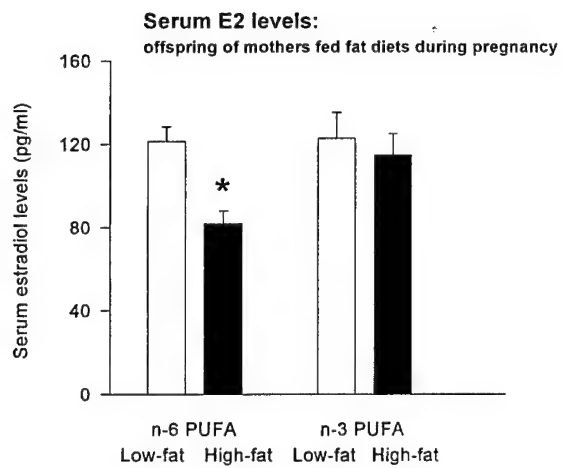
Figure 2.

Hilakivi-Clarke et al.

A

TEBs:
in utero fat exposures

Lobules:
in utero fat exposures

TEBs:
in utero genistein exposures

Lobules:
in utero genistein exposures
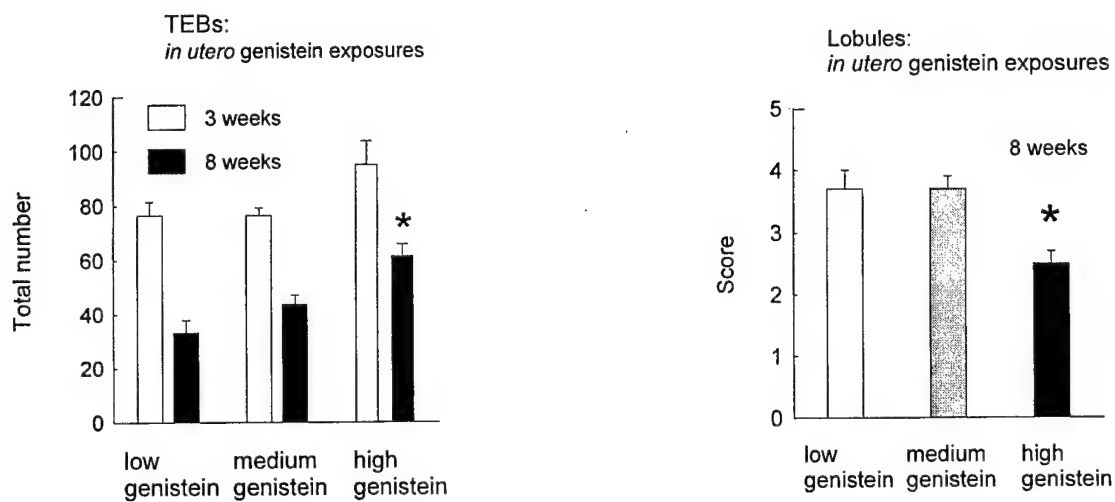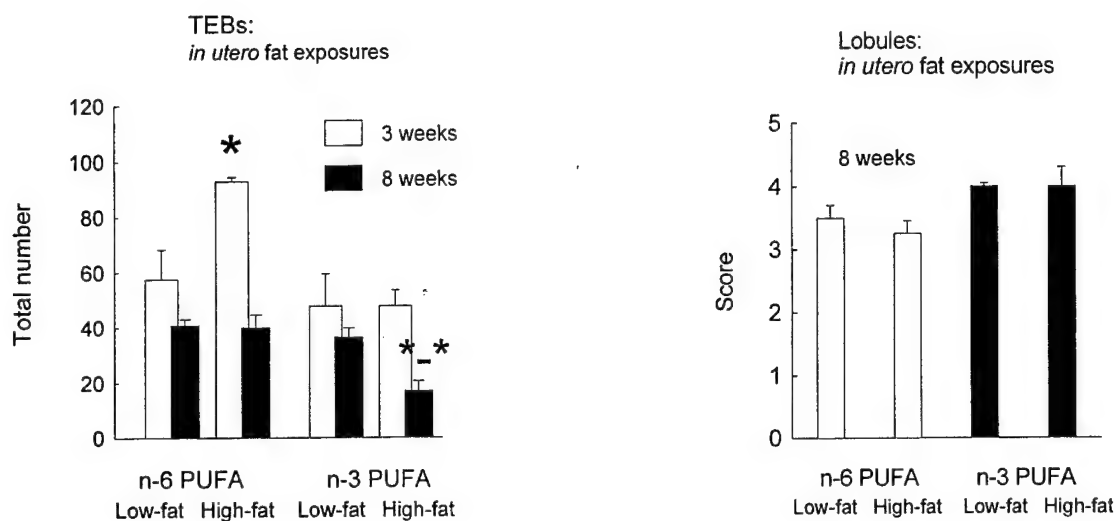
B

Figure 3.
Hilakivi-Clarke et al.

A

DMBA-induced mammary tumor incidence:
in utero n-3 or n-6 PUFA exposures

B

DMBA-induced mammary tumorigenesis:
in utero exposure to genistein
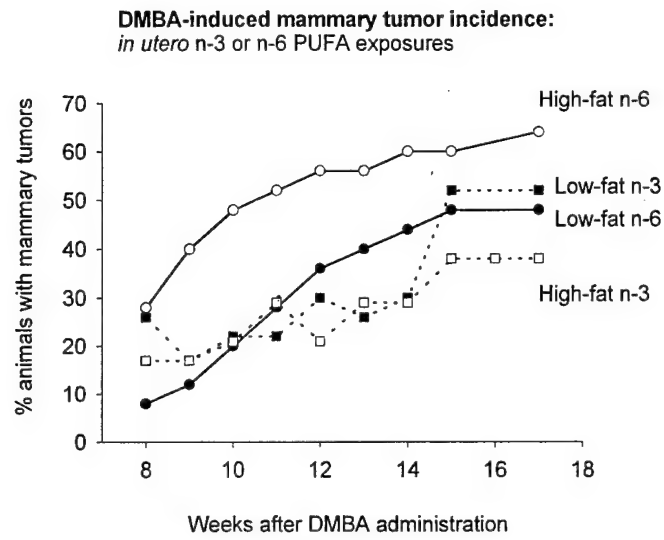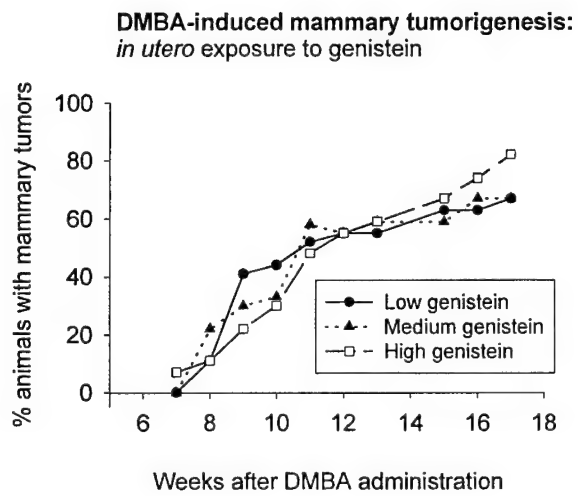

Figure 4.
Hilakivi-Clarke et al.

Soy diet during pregnancy reduces carcinogen-induced mammary tumorigenesis and causes a persistent increase in estrogen receptor β protein levels in the rat mammary gland

Anna Cabanes[1], Sonia DeAssis[1], Dezhong Joshua Liao[1] Jan-Åke Gustafsson[2], Erica Ealley[1], William Helferich[3], and Leena Hilakivi-Clarke[1,3]

[1]Lombardi Cancer Center, and [4]Department of Oncology, Georgetown University, 3970 Reservoir Rd, NW, Washington, DC 20007, USA; [2]Department of Medical Nutrition, Karolinska Institute, Huddinge 14186, Sweden
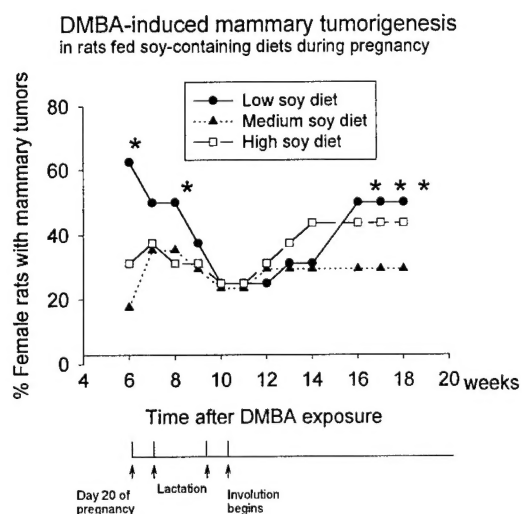
Figure 1. The proportion of female rats exposed to diets containing low (15 mg/kg diet), medium (150 mg) or high (300 mg) genistein levels in soy isolate during pregnancy, that later developed DMBA-induced (administered on day 47) mammary tumors. Tumor incidence was significantly lower in the rats exposed to medium genistein soy diet than in the low genistein soy diet (χ2-test: $p<0.05$).

FIGURE 2

ERα and ERß protein expression in tumor-free mammary glands of rats exposed to high, medium or low genistein levels in soy isolate treated with DMBA 17-weeks earlier.
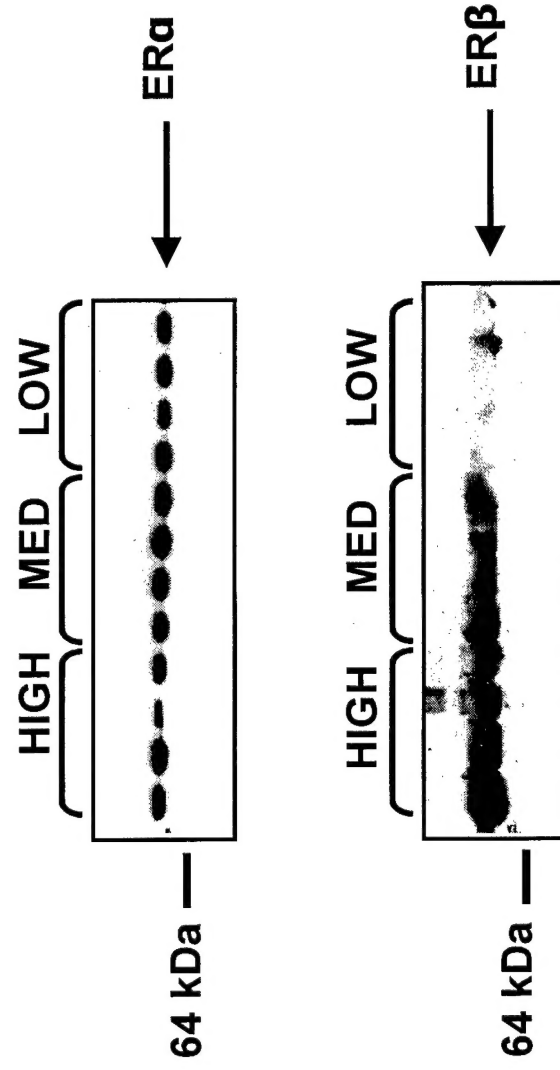
Tumor Biology Program - Life-style and Cancer Prevention

Spring, 2001

Offered by the Tumor Biology Graduate Program

Meeting Place:	W302
Day & Time:	Tuesdays and Thursdays, from 3.30-4.30 p.m.
Format:	The course will consist of presentations primarily by the Faculty and class discussions of any materials provided to the students in advance. Students may be required to read up to 4 papers per week, and to participate in all class discussions.

This is a two credit, advanced course in Cancer Prevention. The course will cover the life-style related risk factors associated with selected cancers with special emphasis on the nutrition, environment, and specific behaviors.

The goals of the course are to (1) provide students with an understanding of the general principles involved in cancer prevention by life-style modifications from both the basic science, clinical and epidemiologic perspectives, (2) develop critical scientific reading and comprehension skills, and to (3) develop interactive skills within a scientific discussion forum.

Grading:	Mean grade from a mid-term and final exams, and final library research paper. For the latter, subjects and mentor to be chosen by students from within course Faculty.
Course Directors:	Leena Hilakivi-Clarke, PhD, Associate Professor of Oncology, and Marc Schwartz, PhD, Assistant Professor.
Information:	Leena Hilakivi-Clarke Lombardi Cancer Center W405A New Research Building Tel: 687-7237

TUMOR BIOLOGY PROGRAM - LIFE STYLE AND CANCER PREVENTION

Part 1: Basic Concepts

		date
1. Introduction to the Course	Hilakivi-Clarke	1/16
2. Basic Concepts of Nutrition	Storey	1/18
3. Basic Concepts of Behavioral Science	Schwartz	1/23
4. Basic Concepts of Chemical Carcinogenesis	Goldman	1/25
5. Basic Concepts of Cancer Epidemiology	Trock	1/30

Part 2: Nutrition and cancer risk

1. Dietary Fats	Hilakivi-Clarke	2/1
2. Calories and Obesity	Storey	2/6
3. Phytoestrogens	Hilakivi-Clarke	2/8
4. Vitamins	Byers	2/13
5. Fiber	Storey	2/15
6. Alcohol	Hilakivi-Clarke	2/20
	No class	2/22
Midterm exam		2/27

Part 3: Other life-style factors and cancer risk

1. Psychosocial Factors and Cancer Risk	Hilakivi-Clarke/Kerr	3/13
2. Environmental Pollutants and Cancer Risk	Trock	3/15
3. Heavy Metals and Cancer Risk	Martin	3/20
4. Physical Activity and Cancer	Audrain	3/22
	No class	3/27 & 3/29
5. Smoking and Cancer Risk	Shields	4/3
Discussions		4/5

Part 4: Cancer risk and behavior

1. Life-style Interventions	Hughes/Audrian	4/10
2. Cancer Risk and Psychosocial Factors	Schwartz	4/17
3. Early Detection	Taylor	4/19
4. Behavioral Aspects of Genetic Testing for Cancer Susceptibility	Schwartz	4/24
5. Sociocultural Aspects of Cancer Prevention and Control	Hughes	4/26 (papers due)
Discussions		5/1
	No class	5/3
Final exam		5/8

Grading

Mid-term exam: A written exam on parts 1 and 2.

Final exam: A written exam on parts 3 and 4. The students will write one essay on one topic selected from any of the subject areas.